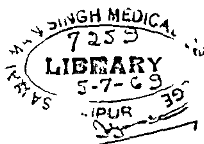
ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA



VOL 74 1968

MUNKSGAARD COPENHAGEN 1968

Munksgaard
International Booksellers and Publishers Ltd
47 Prags Boulevard DK 2300 Copenhagen S Denmark



[illegible]

INDEX

VOL. 74 FASC 1–4 1968

SUPPLEMENTUM

Supplementum 197 Ebbesen P. A Subcellular Agent Inducing Plasma Cell Leukemias in Mice. The relation of this agent to amyloidosis and chronic murine pneumonia. Pp 67. 1968.

Acta path microbiol scandinav 74 1–10 1968

The Institute of Anatomy and Histology Lund the Department of Pathology Malmo and the Department of Pharmacology Lund University of Lund Sweden

ON THE OCCURRENCE OF MONOAMINES AND RELATED SUBSTANCES IN FAMILIAL MEDULLARY THYROID CARCINOMA WITH PHAEOCHROMOCYTOMA

By

BENGT FALCK OTTO LJUNGBERG and EVALD ROSENGREN

Received 15 ii 68

The syndrome medullary thyroid carcinoma associated with phaeochromocytoma has recently been defined as a specific hereditary entity (20 16) The thyroid cancer in this syndrome is of the otherwise rare medullary type described in 1959 by *Ha ard Hawk & Crile* (13) who emphasized the diagnostic significance of amyloid substance in the stroma of this tumour

The histogenesis of the medullary thyroid carcinoma is obscure and the pathogenesis of the syndrome therefore enigmatic Some authors believe that the amyloid material in the thyroid tumour probably consists of thyroglobulin like substances and that the medullary carcinoma may be a thyroid tumour derived from follicular epithelial cells (1 12 21) Autoradiographic studies after the administration of labelled iodine to patients have however failed to demonstrate any radio iodine within the amyloid or the tumour cells (15) On the other hand it is noteworthy that tumour cells displaying a positive chromaffin as well as argentaffin reaction have recently been found in medullary thyroid cancer (*Ljungberg* 1966 unpublished observations) It is therefore possible that the medullary thyroid tumour and the phaeochromocytoma in these patients are in some way related to each other or at least have some causal principle in common This view is possibly supported by the multifocal character of the disease (multiple medullary tumours in one and the same thyroid gland the high incidence of *bilateral* adrenal tumours the occurrence of other neural tumours)

The non acinar parafollicular cells also known as C cells (17) of the thyroid gland which have long been recognized in several mamma

The investigations were supported by grants from the *Swedish Cancer Society* (67–111) and from the *Swedish Medical Research Council* (B68 12X 712 03B and B68 14X 56 04B)

lian species have recently been reported to possess the capacity to form and store certain monoamines. Such amine mechanisms in the parafollicular cells have been demonstrated in many mammalian species including man (10). These findings raise a new possibility of attacking problems concerning the histogenesis and biochemical characteristics of the cells constituting the medullary thyroid cancer.

MATERIAL AND METHODS

Tumours from four patients with the familial syndrome were used (Table 1), viz. two phaeochromocytomas (cases 1 and 4) and three medullary thyroid carcinomas (cases 2, 3 and 4). *The phaeochromocytoma of case 2 could not be made available* for analysis and in case 3 no phaeochromocytoma has been found. All four patients had thyroid tumours, three of which are included in this investigation. The fourth patient (case 1) had been thyroidectomized 3 years before the beginning of the present study. In cases 1, 2 and 4 the thyroidectomy preceded removal of the adrenal tumours by 7 months to 3 years. The three patients with phaeochromocytomas, which were unilateral in all cases (Nos. 1, 2 and 4), had had episodes of bradycardia, pounding headache, peripheral pallor, dyspnoea followed by tachycardia, peripheral vasodilatation and to some extent also sweating. None had sustained arterial hypertension. In 3 patients (Nos. 1, 2 and 4) the urinary excretion of catecholamine metabolites was increased (see Table 3; these analyses were kindly carried out by Dr. *H. von Studnitz* at the Department of Clinical Chemistry, General Hospital, Malmö). The patients in cases 1, 2 and 3 are sisters and the patient in case 4 is a cousin of these. The family has been described in detail elsewhere (16). Apart from local subcutaneous administration of 15–50 ml of 1 per cent carbocaine with adrenaline before thyroidectomy none of the patients were given adrenaline, noradrenaline or related substances in connection with the operations. No adrenaline was found in the subsequent chemical analysis of thyroid gland tissue (see below). Because of a rise in the blood pressure patient 4 was given phentolamine during adrenalectomy. This substance, however, is not known to affect the tissue stores of biogenic monoamines. All four patients were operated upon under general anaesthesia.

Immediately after removal of the tumours representative tissue pieces were quenched in liquid propane cooled by liquid nitrogen. The tissue for chemical analysis was immediately placed in 0.4 N perchloric acid at 0 °C.

In the histochemical investigation the fluorescence method of *Falck & Hillarp* was used. The technical details of this method have been described elsewhere (9). This method allows the demonstration of catecholamines, 5-hydroxytryptamine and some closely related substances at the cellular level. It is based on the principle that in nearly dry environments the amines condense with formaldehyde to form compounds exhibiting a strong fluorescence upon activation with UV light. The fluorophores of the catecholamines emit a green to sometimes (when occurring in high concentrations) yellow-green fluorescence, whereas the fluorophore of 5-hydroxytryptamine exhibits a yellow light under the optical conditions used (9). It is thus possible to distinguish between the catechol derivatives and the indole derivatives. Furthermore secondary catecholamines such as adrenaline need longer treatment in formaldehyde gas to develop maximal fluorescence than do primary catecholamines.

Adrenaline, noradrenaline, dopamine and 5-hydroxytryptamine were assayed by spectrophotofluorimetric methods (2, 3). For routine histology the following stains were used: haematoxylin-erythrosin, Congo red, methyl violet and van Gieson's stain. Every tenth section prepared for fluorescence microscopy was stained with haematoxylin-eosin to facilitate cell identification.

Five cases of benign goitre were studied as control material (Table 2).

RESULTS

Pathological Findings

The thyroid tumours from cases 1–4 were rounded with pale brown slightly bulging cut surfaces Each thyroid studied showed several well demarcated areas of tumour tissue They were often enclosed by a narrow connective tissue zone Here and there were small areas of calcification

Microscopically all four tumours were of the medullary cancer type Some areas showed the features described by *Hazard et al* i e tumour cells of roughly uniform appearance arranged in an irregular trabecular pattern in a connective tissue stroma with varying amounts of amyloid reacting positively with the staining method used Other areas—sometimes even in one and the same thyroid gland—simulated a spindle cell tumour with elongated tumour cells arranged in whirls and streaks In these areas the stroma was sparse and the amount of amyloid material was scanty The neoplastic cells were of fairly uniform appearance with few mitotic figures There was a marked tendency to palisade arrangement of the tumour cells In some areas small uniform cells were arranged in a carcinoid like pattern with rounded clusters of varying size separated by a connective tissue stroma Some of these clusters contained small amounts of amyloid material The marginal cells were often columnar and were arranged in palisade like fashion

The adrenal tumours weighed 67 gr in case 1 27 in case 2 and 1020 in case 4 They were all rounded and encapsulated with small islands of yellow adrenocortical tissue outside the capsule in case 4 small areas of such tissue were also enclosed within the capsular connective tissue In case 1 the tumour was solid in cases 2 and 4 it was cystic The cut surfaces were pale somewhat red brown but in some areas there were degenerative changes with necroses fibrosis and calcification Histologically typical phaeochromocytoma tissue was observed with irregular cells rich in cytoplasm and with rather polymorphous nuclei The cells were usually arranged in small clusters but in some regions they showed an irregular trabecular pattern The stroma was usually sparse but richly vascularized No amyloid substance could be demonstrated in these tumours

The five tumours used as control material included four (cases 5–8) with benign uninodular or multinodular follicular adenomatous goitre one (case 8) associated with Hashimoto s thyroiditis The fifth (case 9) showed granulomatous thyroiditis

Histochemical Investigation

In all four cases of the medullary thyroid cancer a formaldehyde induced green to yellow green flu[illegible] appeared in many tumour cells irregularly distributed among no[illegible]rescent tumour cells (Fig 1 and 2) The proportions of fluorescent and non fluorescent cells vari[illegible]



Fig 1

Medullary thyroid carcinoma of case 4. Carcinoid-like pattern with abundant cells exhibiting a green fluorescence



Fig 2

Medullary thyroid carcinoma of case 4. The strongest fluorescence is displayed by the columnar cells in the margins of the tumour cluster

from one part of the tumour to another The fluorescence which was confined to small cytoplasmic granules did not appear when the formaldehyde treatment was omitted A fairly long exposure (20 min) of the sections to UV light brought about a clearly visible decrease in the intensity of the emitted light Further there was no demonstrable increase in fluorescence intensity or number of fluorescent cells in the specimens that had been subjected to prolonged treatment (3 hrs) in formaldehyde gas There is thus strong reason to believe that the observed fluorescence was derived from a primary catecholamine or some closely related substances (cf 9) No specific *i e* formaldehyde induced fluorescence was ever seen in surrounding normal thyroid tissue except in some adrenergic nerves However in all specimens whether treated in formaldehyde or not abundant cells appeared in the tumours as well as in the surrounding normal thyroid tissue which contained cytoplasmic granules displaying an orange sometimes yellow autofluorescence Only sparse amounts of arterial vessels were seen within the tumour masses In connection with these vessels green fluorescent varicose adrenergic nerves were observed but only few much fewer than ordinarily seen in adrenergic vascular plexus No specific fluorescence could be observed in the amyloid masses that exhibited a very weak dark greenish colour nor did the tumour or the normal thyroid tissue show any specific yellow fluorescence indicating the presence of 5 hydroxytryptamine

In the medullary thyroid tumours of cases 2 and 3 some areas showed the histological picture described by *Hazard et al* (13) In large parts of these areas only unspecific fluorescence was seen but some regions showed unevenly scattered cells with the specific fluorescence described above They seemed to be most numerous in zones close to unaffected thyroid parenchyma but were found also in the central parts of the tumour

In case 4 areas with carcinoid like and spindle cell like patterns were seen In these regions the specific fluorescent cells were also distributed unevenly but where they occurred they were usually very abundant The carcinoid like areas seemed to exhibit the most intense fluorescence here nearly all cells displayed a green to yellow green cytoplasmic fluorescence with varying intensity The marginal columnar cells arranged in the palisade like fashion mentioned above fluoresced more intensely than the cells of the central parts of the clusters (Figs 1 and 2)

In the thyroid material from the five cases of benign goitre no structures developed specific fluorescence except the adrenergic vascular nerves

Examination of the two p[illegible] mas ([illegible]ses 1 and 4) showed as expected an extrem[illegible] [illegible] fluorescence The emitted light was yellow to y[illegible]llow [illegible] which occurs in cells storing large amoun[illegible]s of ca[illegible] [illegible]) Some of th

nuclei appeared non fluorescent, others displayed a weak green fluorescence especially in the nucleoli. The cause of the nucleolar fluorescence is not understood but it may be a diffusion artefact. The interstitial tissue strands showed a strong green fluorescence which like the cellular fluorescence was absent in the tissue pieces not treated with formaldehyde gas. The technique used for processing the tumour tissue for fluorescence microscopy does not seem to allow such an artificious displacement of catecholamines; the phenomenon reflects rather a release of catecholamines from the cells (possibly during the surgical procedure). Sections from material not treated with formaldehyde showed only a very weak greenish fluorescence.

Chemical Investigation

As can be seen from Tables 1 and 2 the presence of 5-hydroxytryptamine was demonstrated in all the medullary thyroid tumours as well as in the phaeochromocytomas investigated, while no 5-hydroxytryptamine could be found in the reference specimens (cases 5–9). The noradrenaline content of the medullary tumours did not differ significantly from that of the reference material. The phaeochromocytomas in cases 2 and 3 contained also dopamine, noradrenaline and adrenaline in concentrations expected in this type of tumour.

TABLE 1

The Content of Catecholamines and 5-Hydroxytryptamine in Phaeochromocytoma and Medullary Thyroid Carcinoma

Case no	Age years	Sex	Type of tumour	Dopamine	Noradrenaline	Adrenaline	5-Hydroxytryptamine
1	49	♀	Phaeochromocytoma	5.6	5010	1330	0.3
			Medullary thyroid carcinoma	Histochemical and chemical examination not performed			
2	59	♀	Phaeochromocytoma	Histochemical and chemical examination not performed			
			Medullary thyroid carcinoma	0	0.14	0	1.03
3	53	♀	Medullary thyroid carcinoma	Only histochemical examination performed			
4	44	♀	Phaeochromocytoma	12.7	3440	1350	0.90
			Medullary thyroid carcinoma	0	0.06	0	0.30

The values are given in μg/g tissue wet weight

TABLE 2

The Content of Catecholamines and 5 Hydroxytryptamine in Five Cases of Benign Goitre

Case no	Age years	Sex	Type of goitre	Dopamine	Noradrenaline	Adrenaline	5 Hydroxytryptamine
5	49	♀	Multiple follicular adenomas + Hashimoto s thyroiditis Possibly slight hyperthyroid	0	0 11	0	0
6	26	♀	Solitary follicular adenoma Euthyroid	0	0 06	0	0
7	59	♀	Multiple follicular adenomas Euthyroid	0	0 24	0	0
8	58	♀	Multiple follicular adenomas Euthyroid	0	0 11	0	0
9	35	♀	Granulomatous thyroiditis Euthyroid	0	0	0	0

The values are given in µg/g tissue wet weight

TABLE 3

Urinary Excretion of 3 Methoxy 4 Hydroxy Mandelic Acid and Metaadrenaline plus Normetaadrenaline

Case no	Age years	Sex	3 Methoxy 4 hydroxy mandelic acid	Metaadrenaline plus Normetaadrenaline §
1	42	♀	11 2–18 6	10 0
2	52	♀	5 4– 7 1	3 0–5 0
3	53	♀	3 8– 4 8	0 4
4	44	♀	33 4–36 6	12 4

All values are given in mg/24 hours
According to Pisano et al (19) Normal range 3 [illegible]–6 6 mg/24 hours
§ According to Pisano (18) Normal range 0–1 2 mg/24 hours

DISCUSSION

The histochemical analyses disclosed the presence of a cell system in the medullary thyroid carcinoma displaying a specific fluorescence (confined to fine cytoplasmic granules) characteristic of primary catecholamines and certain closely related substances According to the chemical analyses however no dopamine or adrenaline could be detected and noradrenaline was found to be present only in amounts equal to those present in the reference cases of benign goitre Moreover the concentrations of noradrenaline were far too low to explain the intense fluorescence exhibited by numerous tumour cells but might on the

other hand well represent the noradrenaline in the adrenergic nerves. As to the reason why the results of the chemical analysis were not in accord with those of the histochemical analysis, the following possibilities deserve attention.

1) The fluorophore is not derived from the catecholamines but from some closely related substance which can condense with formaldehyde to an intensely fluorescent derivative but which is not demonstrable by the chemical methods used. Analysis of the urine from many of the affected members of the family showed that a large part of the urinary catecholamine metabolites consisted of methoxy derivatives demonstrable by the histochemical but not by the chemical method used. Another conceivable possibility is that the fluorescence is not derived from an amine but from a related amino acid. The existence of biological storage mechanisms for amino acids of this type has been questioned, but it has recently been shown that DOPA occurs in considerable quantities in human malignant melanomas (6, 7).

In this connection it should be pointed out that the histochemically demonstrated substance need not be formed in the actual tumour cells. If these cells derive from the parafollicular cell system, they may well possess a mechanism for taking up and concentrating monoamine and their precursors. It has been shown that the parafollicular cells (which normally contain relatively large amounts of 5-hydroxytryptamine in sheep and goats) in mouse thyroid effectively take up both L-DOPA and 5-hydroxytryptophan and decarboxylate them to corresponding amines which are then stored in the cytoplasm by a mechanism that is sensitive to reserpine. Dopamine and 5-hydroxytryptamine and the d-isomers of their precursors pass into the cells to a minor extent (14). Further, it has recently been demonstrated that these amino acids are taken up by parafollicular cells in several mammalian species including man (10). In view of the common co-existence of phaeochromocytoma in the present material such an uptake mechanism *in the medullary thyroid tumour cells cannot be excluded.*

2) Another possibility that should perhaps be borne in mind is that the tumour cells contain some of the known catecholamines but that the amine in the malignant cell is so firmly bound that it cannot be extracted with the method used.

The fact that 5-hydroxytryptamine, which has been shown chemically to occur in medullary thyroid cancer, could not be demonstrated by histochemical methods also calls for an explanation. It is possible that a weak 5-hydroxytryptamine fluorescence may be masked by the yellow autofluorescence or even by the green fluorescence, since a cell may contain more than one biologically active amine (4, 10).

A further possibility is that this amine, which occurs in only relatively small quantities, is diffusely distributed in the tumour and therefore cannot be demonstrated histochemically. *Falck et al.* (unpublished observations) recently showed that some experimental melanomas

contain considerable quantities of DOPA which could be demonstrated chemically but not by histochemical analyses according to the fluorescence method

The findings reported above appear to warrant the following conclusion the fact that 5 hydroxytryptamine was demonstrated chemically and that an extensive cellular system in the medullary thyroid cancer stored a substance in intracytoplasmatic granules which under the histochemical conditions used condensed with formaldehyde with the formation of a fluorescent derivative suggests that monoaminergic mechanisms operate in certain tumour cells in the light of our present knowledge of monoaminergic mechanisms in parafollicular cells this suggests that medullary thyroid cancer may be derived from the non acinar parafollicular cellular system

An unexpected finding was the presence of 5 hydroxytryptamine in the phaeochromocytomas In this respect the phaeochromocytoma in the medullary thyroid carcinoma phaeochromocytoma syndrome seems to differ from other types of phaeochromocytoma not associated with medullary thyroid cancer

SUMMARY

Tumours from four patients with familial medullary thyroid carcinoma associated with phaeochromocytoma were investigated by chemical analysis and the histochemical method of *Falck & Hillarp* for demonstration of certain biogenic monoamines and closely related substances

5-Hydroxytryptamine could be demonstrated chemically in all the medullary thyroid tumours as well as in the phaeochromocytomas investigated while the noradrenaline content of the thyroid cancers did not differ significantly from those of a reference material consisting of five cases of benign goitre

The histochemical analysis disclosed the presence of a cellular system in the medullary thyroid carcinoma displaying a specific granular cytoplasmic fluorescence characteristic of compounds as the primary catecholamines while no fluorescence characteristic of 5 hydroxytryptamine could be observed Possible explanations for this discrepancy between the chemical and the histochemical findings are offered

The fact that 5 hydroxytryptamine could be demonstrated chemically in the medullary thyroid tumours and that numerous cells in these tumours exhibited a specific fluorescence when treated according to the *Falck Hillarp* technique argues for the presence of monoaminergic mechanisms in some of the tumour cells and suggests that medullary thyroid cancer may be derivated fr[illegible]m the pa[illegible]follicular cellular system of the thyroid gland

These findings support the [illegible] mmon principle in the causation of the thyroid and [illegible] this syndrome

RLFERENCES

1 *Albores Saavedra J Rose C C Ibane M I Russel W O Grey C F & Dmo chowski I* The amyloid in solid carcinoma of the thyroid gland Staining characteri tics tis ue culture and electron micrescopic observa tions Lab Invest *13* 77-93 1964

2 *Bertler A Carlsson A Rosengren F & Waldeck B* A method for the fluor imetric determinaticn of adrenaline nora lrenaline and d pamine in tis sues Kungl Fysiogr Sällsk Lund Förh 28 121-123 19 8

3 *Bertler A & Rosengren F* On the distributi n in brain of mcnoamines and of enzymes resp nsible for their formation Experientia *15* 382 384 1959

4 *Cegrell L* Monoamine containing cells in the fetal and newborn guinea pig pancreas Life Sciences 6 1643-16 2 1967

5 *Enemar A Falck B & Håkansson R* Observations on the appearance of nor epinephrine in the sympathetic nervous system of the chick embryo Develop Biol *11* 268 283 1965

6 *Falck B Jacobsson S Olivecrona H & Rorsman H* Pigmented nevi and malignant melanomas as studied with a specific fluorescence method Science *149* 439 440 1965

7 *Falck B Jacobsson S Olivecrona H Olsen G Rorsman H & Rosengren F* Determinati n of catecholamines 5 hydroxytryptamine and 3 4 dihydroxy phenylalanine (Dopa) in human malignant melanomas Acta derm vene reol *46* 65 67 1966

8 *Falck B Larsson B von Mecklenburg C Rosengren F & Svenaeus K* On the presence of a second specific cell system in mammalian thyroid gland Acta physiol scand 62 491-492 1964

9 *Falck B & Owman Ch* A detailed methodological description of the fluor escence method for the cellular demonstration of biogenic monoamine Acta Univ Lund Sectio II No 7 1-23 1965

10 *Falck B & Owman Ch* 5 hydroxytryptamine and related amines in endocrine cell systems Adv in Pharmacol 6 211-231 1968

11 *Falck B & Torp A* A fluorescence method f r hi t chemical demonstration of noradrenaline in the adrenal medulla Med Exp 1 429-432 1961

12 *Freeman D & Lindsay S* Medullary carcinoma of the thyroid gland Arch Path *80* 575-582 1965

13 *Hazard J B Hawk W A & Crile G Jr* Medullary (solid) carcinoma of the thyroid—a clinicopathologic entity J Clin Endocr *19* 152 161 1959

14 *Larsson B Owman Ch & Sundler F* Monoaminergic mechanisms in para follicular cells of the mouse thyroid gland Endocrinology *78* 1109-1114 1966

15 *Ljungberg O* Medullary carcinoma of the human thyroid gland Autoradio graphic localizati n of radioiodine Acta path microbiol scandinav *68* 476-480 1966

16 *Ljungberg O Cederquist F & von Studnitz W* Medullary thyroid carcinoma and phaeochromocytoma A familial chromaffinomatosis Brit Med J *1* 279-281 1967

17 *Pearse A G F* The cytochemistry of the thyroid C cells and their relationship to calcitonin Proc Roy Soc B *164* 478 487 1966

18 *Pisano J J* A simple analysis for normetanephrine and metanephrine in urine Clin Chim Acta 5 406-414 1960

19 *Pisano J J Crout J R & Abraham D Determination of 3 methoxy 4 hydroxy* mandelic acid in urine Clin Chim Acta 7 285-291 1962

20 Schimke R N & *Hartmann W H* Familial amyloid producing medullary thyroid carcinoma and phaeochromocytoma Ann Int Med *63* 1027-1039 1965

21 *Vassar P S & Culling C F* The significance f amyloid in carcinoma of the thyroid gland Am J Clin Path *36* 244 247 1961

Acta path microbiol scandinav 74 11-25 1968

The Department of Pathological Anatomy University of Turku and the Regional Hospital of Rauma Finland

EXTRASKELETAL RETROPERITONEAL OSTEOSARCOMA PROBABLY ARISING FROM MYOSITIS OSSIFICANS

By

O H JÄRVI H T A KVIST and P V VAINIO

Received 15 iii 68

The question whether an extraskeletal osteosarcoma could develop in a myositis ossificans focus has been much discussed It is quite evident that those authors are right who insist that many early cases of myositis ossificans have been misinterpreted as sarcomas by pathologists (*Fine & Stout* 1956 *Ackerman* 1958 *Ackerman & Spjut* 1962 *Gilmer & Anderson* 1959 *Mobius* 1963) In spite of his extensive experience in bone pathology Ackerman has never personally identified a case of myositis ossificans with sarcomatous transformation *Fine & Stout* (1956) however collected 10 cases from the literature and had an additional two of their own in which some support could be found for the hypothesis that a benign tumour might change into one of malignant nature but histological study of the pre existing tumour was done in only one of the reported cases (*Coley* 1913) Earlier *Shipley* (1940) had collected four cases of development of osteosarcoma in pre existing myositis ossificans not referred by *Fine & Stout* *Uehlinger* (1955) and *Collins* (1966) share the opinion that osteosarcoma may develop from myositis ossificans

In the following an osteosarcoma case is reported in which the most probable explanation of its origin is development from a myositis ossificans focus The case also attracts attention because of the location of the tumour in retroperitoneal tissue The great majority of extra skeletal osteosarcomas described have been located to the extremities

CASE REPORT

The patient a 55 year old charwoman had been examined earlier on March 17 1960 in the out patient department because of upper abdominal pain The roentgenograms revealed gallstones and in addition a mottled calcification nearly 2 cm in diameter at the level of the XI left intercostal space close to the spine (Fig 1A)

Because of a mass in the left upper abdomen the patient was admitted in August

Address for reprints Dr O H Järvi Department of Pathological Anatomy University of Turku Kiinamyllynkatu 10 Turku 3 Finland



Fig 1 4

Roentgenogram March 7 1960 The contrast filled stomach shows no abnormality Behind the stomach is a partially observable round calcified body (arrows)

1967 to the department of surgery at the Regional Hospital at Rauma She had noticed the slowly growing tumour some three months earlier and had experienced dorsally radiating pain low in the abdomen on lifting There were no irregularities in bowel function or micturition

On admission the patient was in good general condition with normal heart and lungs The sedimentation rate was 21 mm/h the haemoglobin 13.2 mg per cent and the differentiated blood count normal Save for albuminuria the urinalysis was non contributory The serum creatinine level was 0.75 mg per cent A firm non tender tumour the size of a child's head was felt in the left upper abdomen and there was left sided tenderness on percussion in the back.

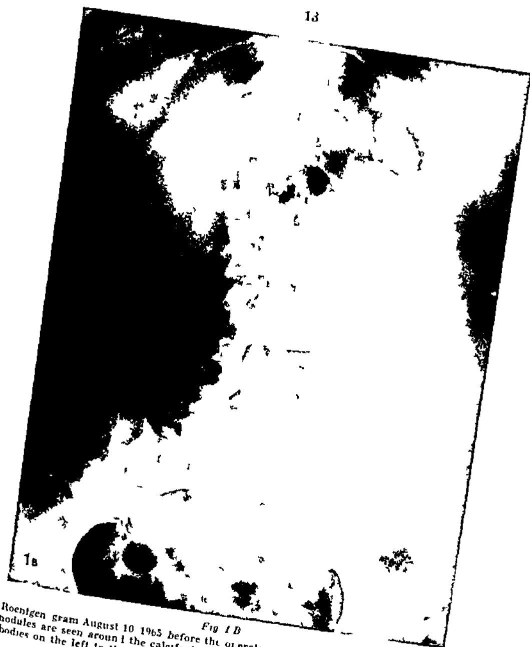


Fig 1 B

Roentgen gram August 10 1965 before the operation for the tumour Several smalle nodules are seen aroun l the calcifie l l ls in Fig 4 The tumour extend from the bodies on the left to the pelvic cavity f r further inf rmati n ee text Call tone are en t the right

Roentgen grams (Fig 1B) aga n 4 × × 2 a cm whi h had b n t 5 cm to the left of the midlie lower a second less distin tl tected These calcification peritoneally and were surr

d he c l c l ium dep s t me ur ng he l n 960 and which was located l r he t lft thorac vertebra Slightly a ft t n 1 8 × 1 4 × 1 0 cm wa d a b l the diaphragm and retr u lc fi l granules From



Fig 1C

Roentgenogram November 11 1965 after removal of the main bulk of the tumour. The calcified bodies in the upper retroperitoneal tissue are still visible

posits a soft tissue shadow extended into the region of the left psoas muscle and kidney. Diffuse striation and blotchiness indicated also a possible calcification of this tumour. A barium meal revealed an otherwise normal stomach but the angulus was dislodged to the right because of external compression. An intravenous pyelogram disclosed slightly deformed lower calyces on the left and some compression of the left ureter.

With a pre-operative diagnosis of a left renal tumour laparotomy was performed on August 19 1965. The 20 cm long smooth easily bleeding tumour however was



Fig 2
The removed tumour cleaved

not of renal origin but was firmly attached to the posterior abdominal wall from the lower ribs to the iliac fossa and likewise on its ventral and left surface to the transverse and descending colon The idea of radical removal of the tumour was abandoned and only a biopsy was performed The histological diagnosis established on the basis of the removed specimen was Fibroma ossificans (Fibrosarcoma?)

As the malignancy thus remained uncertain a further attempt to radically remove the tumour was decided on especially as further growth probably would obstruct the colon On September 3 1965 the tumour was removed and although no macroscopical infiltration of tumour tissue into the intestinal wall could be seen the most adherent part (ca 30 cm) of the colon was simultaneously excised The stomach and the rest of the intestines were not attached to the tumour nor was it adherent to the spine ribs aorta or any parenchymal organs No large vessels entered the tumour but there was massive diffuse haemorrhage Only dissection of the tumour from the upper retroperitoneum was difficult the latter seemed also to be the point of its origin Here a ragged area remained after removal of the tumour and postoperative roentgenograms revealed residual calcified deposits in this area

The removed tumour weighing 2870 g was almost the size of a soccer ball The surface was fairly smooth and covered by a thin capsule It was possible to cut the tumour with a knife although it was reminiscent of cancellous bone and contained several very hard areas (Fig 2)

The patient made an uneventual recovery On September 17 the following laboratory values were recorded ST 98 mm/h Hgb 10 6 mg per cent alkaline phosphatase 4 65 B L units acid phosphatase 0 50 B L units serum calcium 8 0 mg per cent X ray examination on November 11 revealed increased blotchiness in the area of the residual calcified deposits in the upper abdomen (Fig 1C)

In December 1965 the patient was re admitted because of deterioration of her general health and loss in weight Palpation of the abdomen revealed an extensive firm mass obviously a local recurrence The patient expired on January 20 1966

At autopsy nothing abnormal was found in the brain and the cervical and thoracic organs There were extensive adhaesions between the intestinal loops in the abdominal cavity and sanguinolent fluid on the right side A uniform plate of



Fig 3

The abdominal situs at autopsy January 2[illegible] 1966 Bony tumour masses extend from left upper part of the retroperitoneal space along the visceral and parietal peritoneum and over the midline and as far as the pelvis Several darkstained sanguinolent cysts are seen in the tumour tissue For further details see text

tissue hard as bone protruded from below the costal arch on the left extending transversely to the right about 7–8 cm across the median line (Fig 3) It followed the omentum and the mesentery deep to the root Similar bony tumour tissue was demonstrated in association with the parietal peritoneum on the abdominal surface of the diaphragm in the porta hepatis and on the anterior surface of the gastric serosa which also displayed large vesicles containing blackish red blood Tumour



Fig 4

Peritoneal tumour tissue In the upper part parallelly running osteoid trabeculae in the lower part spongy rather well differentiated bone Haematoxylin—van Gieson × 20

tissue was most abundant in the area of the bursa omentalis which it appeared to fill completely

The tumour continued from there to the vicinity of the pancreas and the left adrenal gland but these organs were well preserved The spleen too was associated closely with tumour tissue but it was possible to expose its capsule almost intact The spleen weighed ? 0 g Induration and some bony nodules were encountered also in the region of the uterine ligaments No infiltrations had been established in the muscles except in the diaphragm and there too the relationship with the muscular tissue was uncertain macroscopically The tumour tissue was cancellous on the section surface fairly soft yet distinctly ossified in the marginal parts A plum sized n le which almost t uched on the diaphragm was observed above the left kidney orresponding to the roundish shadow established in the roentgenogram The n de was bony surrounded by fatty tissue and lying separate from the tumour tissue and the organs of the abdominal cavity A smaller bean size l similar body was situated cl se to the previous one The tumour tis ue was not directly con nected anywhere with the ribs spine or other bony parts although it closely ap proa hed the vertebrae There was no connection between the kidneys and the tumour The kidneys were of normal structure and weighed 130 g each

HISTOLOGY

The first biopsy specimen (18 8 6a) was small and hard to interpret Tentative diagnosis Fibroma ossificans or fibrosarcoma with osteoid elements

The specimens from the tumour removed at operation and at autopsy gave identical pictures Bone formation was widely distributed



Fig 5

Another site of peritoneal intramembranous osteoid and bone formation The lamellar structure of the original peritoneum is evident In the lower part fibrous sarcomatous tissue Haematoxylin—van Gieson × 60

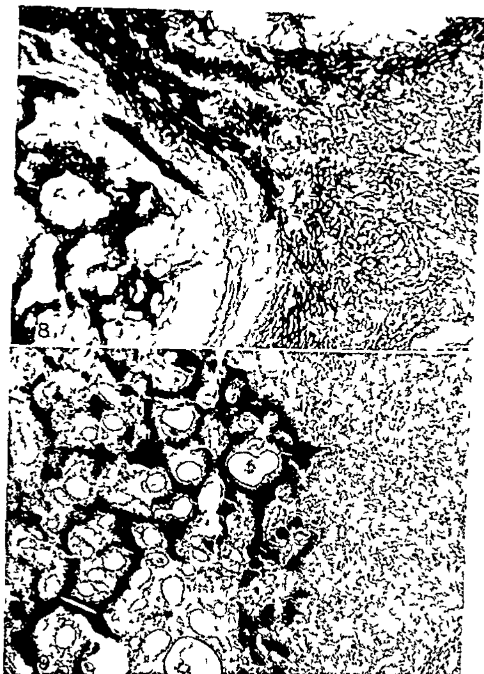
in most specimens It always involved a direct transformation of collagen to bone cartilage never occurred In the peritoneum there were often parallelly running osteoid or bony trabeculae which in the deeper layers were often replaced by a more spongy organized and partly calcified bone (Figs 4 and 5) Both bone and osteoid stained with periodic acid Schiff (PAS) method but with Alcian blue (acetic acid 3 per cent) only osteoid bone gave a positive staining The opposite result was achieved with von Kossa's method for demonstrating calcium Cellular tumour tissue was situated between the trabeculae The cells were mostly spindle shaped sometimes rounded and there was fine reticulin but only scanty collagen and no elastic fibres between the cells A definite but not very marked atypia in the size and shape of the nuclei with a certain amount of hyperchromasy was observable Mitoses were found in moderate numbers Both PAS stain and Best's



Figs 6–7

Fig 6 Cellular fibrous sarcoma tissue with osteoclast type giant cells In the upper part necrosis Haematoxylin—van Gieson × 90

Fig 7 Sinusoid veins with trabecular bone network around the vessels The bone and medullary connective tissue show no signs of malignancy Small accumulation of osteoclasts in connection of trabeculae (arrows) Haematoxylin—Eosin × 30



Figs 8 9

Fig 8 Transformation of the lamellar cancellous bone with fatty marrow into osteoid cellular tumour tissue Haematoxylin—van Gieson × 60

Fig 9 Transformation of the cancellous non malignant bone into cellular sarcoma tissue Haematoxylin—Eosin × 60

carmin staining elicited abundant glycogen granules in the tumour cells particularly in osteoblasts around the bony trabeculae. In places in the tumour tissue large venous sinusoids were noted and often numerous multinuclear giant cells of osteoclast type were detected outside their wall or there were solid cellular areas with giant cells. No atypia was found in the nuclei of the giant cells (Fig. 6). Small Alcian blue positive granules were often observed in their plasma, perhaps deriving from osteoid material. Sometimes the sinusoids were surrounded by a regular fine bone network of highly differentiated structure (Fig. 7). In other areas bone with the beginning of lamellar apposition was observable (Figs. 8 and 9). In these more differentiated areas the marrow could be quite scanty or it might even have changed to fatty tissue (Fig. 8). On the other hand there were areas with haemorrhagic and necrotic cysts and cellular and vascular tissue without bone structure. Multinuclear giant cells could occur in marked numbers among the spindle shaped cells (Fig. 6), suggesting that the areas possibly had developed by osteolysis of the already formed osteoid or bone.

The two pieces of bony tissue found at autopsy and lying separately in the region of the left XI rib were also examined histologically. They consisted of fully mature compact or cancellous lamellar bone (Figs. 10 and 11). No direct transformation to tumour tissue could be verified in these places. In one place there was periosteal activity without clear tumorous atypia. The marrow consisted of thin fibrous collagen or fat tissue. In the actual tumour tissue, however, the transformation of highly differentiated bone to sarcomatous tissue was clearly observed (Figs. 8 and 9).

DISCUSSION

It is difficult to classify the tumour presented here. Its histology is that of osteosarcoma partly with high cellularity and osteoid bone formation; in places the tumour has some resemblance to a vascular giant cell tumour but differs from this in the abundance of bony structure in other places. Clinically as well as anatomically the malignancy of the tumour is quite evident. The rapid progression of growth after the primary removal resulting in the death of the patient eight months and a half after the first subjective symptoms leaves no room for doubt.

The difficulty lies in the origin of the tumour. At the operation it seemed to be attached to the retroperitoneal tissue high on the left just at the site where calcified bodies had been demonstrated by roentgenography in 1960. Macroscopically and histologically, these bodies could be explained most probably as old foci of myositis ossificans in spite of the absence of any report of trauma in the history. According to Ackerman and Spjut only about one half of the myositis ossificans cases have a history of trauma. In the later roentgenograms (August 1965)





Figs 10–11

Fig 10 Compact lamellar *bone* from the retroperitoneal calcified body seen in Figs 1A, B and C Haematoxylin–Eosin × 30

Fig 11 More cancellous lamellar *bone* from the retroperitoneal calcified body second in size seen in Figs 1B and C *The cement* lines of mosaic pattern are distinct *Haematoxylin*—Eosin × 30

when a palpable tumour had already developed most of the calcified foci were concentrated in this area In the recurring tumour (roentgenograms October 1965) the same course of events could be established Histologically areas could be found in the tumour tissue which were difficult to distinguish from what is considered as myositis ossificans There was osteoid and bone formation with minimal atypia alternating with cellular vascular giant cell areas as described by *Fine & Stout* (1956) *Ackerman* (1958) *Ackerman & Spjut* (1962) *Gilmer & Anderson* (1959) and *Mobius* (1963) in their pseudosarcomatous myositis ossificans cases Of course the zonal grouping of these elements in a large tumour like the present one could not have the same regular occurrence as in the benign tumours described by the authors cited

Another alternative might be a mesodermal mixed tumour (mesenchymona) or a teratoid tumour in which bone should be one of the tumour constituents However no other tissue mesodermal or epithelial could be demonstrated The authors therefore rejected these alternatives *and considered the final diagnosis to be an osteosarcoma arising from* myositis ossificans

Myositis ossificans is a rare occurrence in the abdominal wall the retroperitoneal tissue or the lumbar muscular region *Strauss* found none among his 127 cases of myositis ossificans *Geschickter & Maseritz* (1938) reported one in 25 cases *Ackerman* (1958) two out of 26 cases *Ackerman & Spjut* reported one further case in 1962 *Gilmer & Anderson* (1959) one out of 23 cases and *Mobius* one case in 1963 To the best of our knowledge no cases of an osteosarcoma arising from myositis ossificans in this region has been published

Extraskeletal osteosarcomas in the region in question are also very rare *Fine & Stout* (1956) mentioned in their survey of the literature that out of 34 malignant extraskeletal soft part tumours (not located in organs) described four were situated in the abdominal wall or the lumbar region among their own 12 cases one was located in the retroperitoneal space *Lowry & Haynes* (1964) described an osteosarcoma observed within the psoas muscle in a 68 year old man The predominant cells were spindle shaped but monster giant cells also occurred occasionally There were osteoid well developed bony trabeculae and well differentiated cartilage within the tumour The tumour recurred after operation and the patient died 11 months after onset of the symptoms *Lowry & Haynes* also reported that only three out of the 41 cases of extraskeletal osteosarcoma recorded at the Armed Forces Institute of Pathology Washington three originated in the retroperitoneal area *Auerbach et al* (1951) reported osteosarcoma in lumbar soft tissue after irradiation and *Boyer & Navin* (1963) presented a similar case together with another diagnosed as chondrosarcoma with osteogenic elements all occurring in the irradiated field after treatment for a malignant testicular tumour In none of these was a myo

sitis ossificans origin suggested. In the literature concerning retroperitoneal tumours *Donnelly* (1946) mentioned the occurrence of bony or calcareous structures in a few of his 95 cases. *Kundt & Schulte-Brinkmann* (1960) found none among their 30 cases.

In Finnish studies of retroperitoneal tumours *Kalima* (1913, 2 cases), *Lahtenmaki* (1951, 11 cases), *Ahvenainen et al.* (1954, 30 cases), *Paalanen* (1956, 1 case) and *Lofgren* (1967, 17 cases), the last author described one fibrosarcoma case with osseous metaplasia and two chondrosarcoma cases in one of which there was also osteoid and rather well developed bony trabeculae with loose fibrous marrow. The last two fatal cases, originally examined in this laboratory, were not suitable for further study because only a small piece of each of the large tumours was available for microscopic investigation.

The present authors agree with *Line & Stout* in their statement that if myositis ossificans might be the origin of osteosarcoma, the number of such developments must be very small in relation to the large number of occurrences of myositis ossificans cases unassociated with this change. They added, however: "Perhaps the relative number of cases of osteogenic sarcoma developing in myositis ossificans would have been greater if so many of them had not been ablated for surgery." The consequences of traumas in the retroperitoneal space and deep in the back may remain unnoticed and could after a longer period of quiescence lead to a serious disease. A parallel process of events could at least be inferred in the three cited cases of retroperitoneal osteosarcoma developed after irradiation. When the time span between the cause and malignization is long, the real aetiology must in many cases remain obscure in the patient's history, and when he finally comes for consultation the original focus may already be masked by the developed tumour.

Another point which attracts attention in the present case is the difficulty facing the pathologist when he has to anticipate the clinical course, benign or malignant, pseudosarcomatous or sarcomatous, from biopsy specimens. Often several specimens are certainly needed for a correct diagnosis.

SUMMARY

A fatal case of extraskeletal osteosarcoma in the retroperitoneal space is reported. The tumour is thought to have arisen from an old myositis ossificans focus demonstrated at roentgenography five years earlier. The difficulties of histological grading of the malignancy are discussed.

REFERENCES

1. *Ackerman, L. V.*: Extra-osseous localized non-neoplastic bone and cartilage formation (so-called myositis ossificans). J. Bone and Joint Surg. 40 A: 279–298, 1958.
2. *Ackerman, L. V. & Spjut, H. J.*: Tumors of bone and cartilage. Atlas of Tumor Pathology. Section II, Fasc. 4, 1962.

3 *Ahtenainen E K Malmio K & Sulamaa M* Massive retroperitoneal tumors in children Ann Chir Gynaec Fenn 43 1 16 1954
4 *Auerbach O Friedman M Weiss L & Amory H I* Extraskeletal osteogenic sarcoma arising from irradiated tissue Cancer 4 1095–1106 1951
5 *Boyer C W Jr & Navin J J* Extraskeletal osteogenic sarcoma A late complication of radiation therapy Cancer 18 628 633 1965
6 *Coley W B* Myositis ossificans traumatica A report of three cases Ann Surg 57 305 337 1913
7 *Collins D* Pathology of bone London Butterworths 1966 (p 81)
8 *Donnelly B A* Primary retroperitoneal tumors Surg Gynec Obstet 83 705–717 1946
9 *Fine G & Stout A P* Osteogenic sarcoma of the extraskeletal soft tissues Cancer 9 1027–1043 1956
10 *Geschickter C F & Maseritz* Myositis ossificans J Bone and Joint Surg 20 661–674 1938
11 *Gilmer W S & Anderson L D* Circumscribed myositis ossificans Southern Med J 52 1432 1448 1959
12 *Kalima T* Über retroperitoneale Lipome Finska Läk sällsk Handl 10 283 396 1912
13 *Kundt H W & Schulte Brinkman W* Primare retroperitoneale Tumoren Bruns Beitr Klin Chir 201 337–369 1960
14 *Fourg K & Haynes C D* Osteogenic sarcoma of extraskeletal soft tissue A case report Amer Surg 30 97–100 1964
15 *Lähteenmäki S* Uber die Pathologie und Klinik der retroperitonealen mesodermalen Mischtumoren Acta path microbiol scandinav Suppl 61 1945
16 *Löfgren F* Primary retroperitoneal tumours Ann Acad Sci Fenn (Med) Series A V 129 1967
17 *Möbius G* Zur Pathologie der Myositis und Periostitis ossificans Chirurg 34 308 314 1963
18 *Paalanen A* Malignant retroperitoneal leiomyoma Ann Chir Gynaec Fenn 45 77 84 1956
19 *Shipley A M* Ossifying hematoma and allied conditions Arch Surg 41 516 524 1940
20 *Strauss* cited by Lewis D Myositis ossiciacans J Amer Med Ass 80 1281–1287 1923
21 *Uehlinger E & Putscher W* Knochenseminar b 39 Tagg der Deutsch Ges f Path 1955

sitis ossificans origin suggested. In the literature concerning retroperitoneal tumours *Donnelly* (1946) mentioned the occurrence of bone or calcareous structures in a few of his 95 cases. *Kundt & Schulte-Brinkmann* (1960) found none among their 30 cases.

In Finnish studies of retroperitoneal tumours *Kalima* (1913, 2 cases), *Lähteenmäki* (1915, 11 cases), *Ahvenainen et al.* (1954, 30 cases), *Paalanen* (1956, 1 case) and *Löfgren* (1967, 17 cases) the last author described one fibrosarcoma case with osseous metaplasia and two chondrosarcoma cases in one of which there was also osteoid and rather well developed bone trabeculae with loose fibrous marrow. The last two fatal cases, originally examined in this laboratory, were not suitable for further study because only a small piece of each of the large tumours was available for microscopic investigation.

The present authors agree with *Lane & Stout* in their statement that if myositis ossificans might be the origin of osteosarcoma the number of such developments must be very small in relation to the large number of occurrences of myositis ossificans cases unassociated with this change. They added however: "Perhaps the relative number of cases of osteogenic sarcoma developing in myositis ossificans would have been greater if so many of them had not been ablated for surgery." The *consequences of traumas in the retroperitoneal space and deep in the* back may remain unnoticed and could after a longer period of quiescence lead to a serious disease. A parallel process of events could at least be inferred in the three cited cases of retroperitoneal osteosarcoma developed after irradiation. When the time span between the cause and malignization is long, the real aetiology must in many cases remain obscure in the patient's history and when he finally comes for consultation the original focus may already be masked by the developed tumour.

Another point which attracts attention in the present case is the difficulty facing the pathologist when he has to anticipate the clinical course, *benign or malignant, pseudosarcomatous or sarcomatous, from* biopsy specimens. Often several specimens are certainly needed for a correct diagnosis.

SUMMARY

A fatal case of extraskeletal osteosarcoma in the retroperitoneal space is reported. *The tumour is thought to have arisen from an old myositis* ossificans focus demonstrated at roentgenography five years earlier. The difficulties of histological grading of the malignancy are discussed.

REFERENCES

1. *Ackerman, L. V.*: Extra-osseous localized non-neoplastic bone and cartilage formation (so-called myositis ossificans). J. Bone and J. Surg. 40-A: 279–298, 1958.
2. *Ackerman, L. V. & Spjut, H. J.*: Tumors of bone and cartilage. Atlas of Tumor Pathology, Section II, Fasc. 4, 1962.

3 *Ahvenainen E K Malmio K & Salamaa M* Massive retroperitoneal tumors in children Ann Chir Gynaec Fenn 43 1–16 1954

4 *Auerbach O Friedman M Weiss L & Amory H I* Extraskeletal osteogenic sarcoma arising from irradiated tissue Cancer 4 1095–1106 1951

5 *Boyer C W Jr & Navin J J* Extraskeletal osteogenic sarcoma A late complication of radiation therapy Cancer 18 628 633 1965

6 *Coley W B* Myositis ossificans traumatica A report of three cases Ann Surg 57 305–337 1913

7 *Collins D* Pathology of bone London Butterworths 1966 (p 81)

8 *Donnelly B A* Primary retroperitoneal tumors Surg Gynec Obstet 83 705–717 1946

9 *Fine G & Stout A P* Osteogenic sarcoma of the extraskeletal soft tissues Cancer 9 1027–1042 1956

10 *Geschickter C F & Maseritz I* Myositis ossificans J Bone and Joint Surg 20 661–674 1938

11 *Gilmer W S & Anderson L D* Circumscribed myositis ossificans Southern Med J 52 1432–1448 1959

12 *Kalima T* Über retroperitoneale Lipome Finska Läkaresällsk Handl 70 283 306 1912

13 *Kundt H W & Schulte Brinkman W* Primäre retroperitoneale Tumoren Bruns Beitr klin Chir 201 337–369 1960

14 *Loury K & Haynes C D* Osteogenic sarcoma of extraskeletal soft tissue A case report Amer Surg 30 97–100 1964

15 *Lahteenmäki S* Über die Pathologie und Klinik der retroperitonealen mesodermalen Mischtumoren Acta path microbiol scandinav Suppl 61 1945

16 *Lofgren L* Primary retroperitoneal tumours Ann Acad Sci Fenn (Med) Series A V 129 1967

17 *Mobius G* Zur Pathologie der Myositis und Periostitis ossificans Chirurg 34 308–314 1963

18 *Paalanen A* Malignant retroperitoneal leiomyoma Ann Chir Gynaec Fenn 45 77–84 1956

19 *Shipley A M* Ossifying hematoma and allied conditions Arch Surg 41 516–524 1940

20 *Strauss* cited by Lewis D Myositis ossificacans J Amer Med Ass 80 1281–1287 1923

21 *Uehlinger E & Putscher W* Knochenseminar b 39 Tagg der Deutsch Ges f Path 1955

sitis ossificans origin suggested. In the literature concerning retroperitoneal tumours *Donnelly* (1946) mentioned the occurrence of bony or calcareous structures in a few of his 95 cases. *Kundt & Schulte-Brinkmann* (1960) found none among their 30 cases.

In Finnish studies of retroperitoneal tumours *Kalima* (1913, 2 cases), *Lahteenmäki* (1945, 11 cases), *Ahvenainen et al.* (1951, 30 cases), *Paalanen* (1956, 1 case) and *Löfgren* (1967, 47 cases) the last author described one fibrosarcoma case with osseous metaplasia and two chondrosarcoma cases in one of which there was also osteoid and rather well developed bony trabeculae with loose fibrous marrow. The last two fatal cases, originally examined in this laboratory, were not suitable for further study because only a small piece of each of the large tumours was available for microscopic investigation.

The present authors agree with *Lane & Stout* in their statement that if myositis ossificans might be the origin of osteosarcoma the number of such developments must be very small in relation to the large number of occurrences of myositis ossificans cases unassociated with this change. They added, however: "Perhaps the relative number of cases of osteogenic sarcoma developing in myositis ossificans would have been greater if so many of them had not been ablated for surgery." The consequences of trauma in the retroperitoneal space and deep in the back may remain unnoticed and could after a longer period of quiescence lead to a serious disease. A parallel process of events could at least be inferred in the three cited cases of retroperitoneal osteosarcoma developed after irradiation. When the time span between the cause and malignization is long, the real aetiology must in many cases remain obscure in the patient's history, and when he finally comes for consultation the original focus may already be masked by the developed tumour.

Another point which attracts attention in the present case is the difficulty facing the pathologist when he has to anticipate the clinical course, benign or malignant, pseudosarcomatous or sarcomatous, from biopsy specimens. Often several specimens are certainly needed for a correct diagnosis.

SUMMARY

A fatal case of extraskeletal osteosarcoma in the retroperitoneal space is reported. The tumour is thought to have arisen from an old myositis ossificans focus demonstrated at roentgenography five years earlier. The difficulties of histological grading of the malignancy are discussed.

REFERENCES

1. *Ackerman, L. V.*: Extra-osseous localized non-neoplastic bone and cartilage formation (so-called myositis ossificans). J. Bone and Joint Surg. 40-A: 279-298, 1958.
2. *Ackerman, L. V. & Spjut, H. J.*: Tumors of bone and cartilage. Atlas of Tumor Pathology, Section II, Fasc. 4, 1962.

3 *Ahvenainen F K Malmio K & Sulamaa M* Massive retroperitoneal tumors in children Ann Chir Gynaec Fenn 43 1-16 1954
4 *Auerbach O Fiedman M Weiss L & Amory H I* Extraskeletal osteogenic sarcoma arising from irradiated tissue Cancer 4 1095 1106 1951
5 *Boyer C W Jr & Navin J J* Extraskeletal osteogenic sarcoma A late complication of radiation therapy Cancer 18 628-633 1965
6 *Coley W B* Myositis ossificans traumatica A report of three cases Ann Surg 57 305-337 1913
7 *Collins D* Pathology of bone London Butterworths 1966 (p 81)
8 *Donnelly B A* Primary retroperitoneal tumors Surg Gynec Obstet 83 105-717 1946
9 *Fine C & Stout A P* Osteogenic sarcoma of the extraskeletal soft tissues Cancer 9 1025-1042 1956
10 *Geschickler C F & Maseritz* Myositis ossificans J Bone and Joint Surg 20 661-674 1938
11 *Gilmer W S & Anderson I D* Circumscribed myositis ossificans Southern Med J 52 143 -1448 1959
12 *Kalima T* Uber retroperitoneale Lipome Finska Läkaresällsk Handl 10 283 326 1912
13 *Kundt H W & Schulte Brinkman W* Primare retroperitoneale Tumoren Bruns Beitr Klin Chir 201 337-369 1960
14 *Lowry K & Haynes C D* Osteogenic sarcoma of extraskeletal soft tissue A case report Amer Surg 30 97-100 1964
15 *Lahteenmaki S* Uber die Pathologie und Klinik der retroperitonealen mesodermalen Mischtumoren Acta path microbiol scandinav Suppl 61 1945
16 *Löfgren I* Primary retroperitoneal tumours Ann Acad Sci Fenn (Med) Series A V 129 1967
17 *Mobius G* Zur Pathologie der Myositis und Periostitis ossificans Chirurg 34 308-314 1963
18 *Paalanen A* Malignant retroperitoneal leiomyoma Ann Chir Gynaec Fenn 45 77-84 1956
19 *Shipley A M* Ossifying hematoma and allied conditions Arch Surg 41 516-524 1940
20 *Strauss* cited by Lewis D Myositis ossificans J Amer Med Ass 80 1281-1287 1923
21 *Uehlinger F & Putscher W* Knochenseminar b 39 Tagg der Deutsch Ges f Path., 1955

Acta path. microbiol. scandinav. 74: 26–31, 1968

Kaptein W. Wilhelmsen og Frues Bakteriologiske Institutt, University of Oslo and Rikshospitalet, Oslo, Norway

TRANSFERABLE DRUG RESISTANCE OF ENTERIC BACTERIA ISOLATED FROM CLINICAL SOURCES

By

EINAR H. AANDAHL

Received 21.ii.68

The transfer of multiple drug resistance between strains of gram negative rods was originally described in Japan in 1959 (20). Investigations into the epidemiology of bacillary dysentery and the problem of the increase of strains of *Shigella* resistant to multiple drugs led to the discovery of infectious drug resistance which was transferable from one strain to another, rendering the previous sensitive strain simultaneously resistant to several unrelated drugs. An extensive work has been done on the epidemiology and the genetic analysis of the responsible agents which have been termed resistance factors or R factors (14).

There is evidence that the R factors are extrachromosomal genetic elements which possess the property of autonomous replication. They have been identified as consisting of DNA. An R factor consists of a variable number of genetic markers, each conveying resistance to a single antibacterial drug when present in a cell. These resistance markers are linked to another genetic element, the resistance transfer factor, RTF, which imparts to the cell the property of conjugation with transfer of DNA between the conjugation cells. Regarding this property RTF may be considered analogous to the sex factor F of *E. coli* and in particular F′, and thus belongs among the conjugation factors (12). There is evidence that RTF and the resistance determinants exist in a single linear linkage group (14). Spontaneously and during conjugation segregation may occur, producing clones which have lost some or all of the resistance traits (22). The R factors thus exhibit some of the properties ascribed to genetic elements of episomic nature (9). Some workers claim to have proved the existence of a stable integrated state of the R factors in the host chromosome. This is considered indispensable to the episomal concept. More recent investigations have not produced conclusive evidence of the integrated state. The R factors may be referred to as bacterial plasmids (10).

The R factors isolated from *Shigella* strains in Japan transferred resistance to four drugs or less: sulphonamides, streptomycin, chlor

amphenicol and tetracycline Since then transferable drug resistance has been demonstrated in many species of Enterobacteriaceae and from many parts of the world (3) R factor carrying strains have been isolated both from healthy human beings and animals (18 19) and from clinical cases (3 16 17) Some of the R factors confer resistance to seven drugs viz kanamycin neomycin and ampicillin in addition to the above mentioned four (3)

In 1960 a hospital strain of *E coli* which carried a plasmid conveying resistance to low levels of streptomycin only (7) was isolated at this institute Recent investigations have made evident that this plasmid which in addition to the single resistance determinant causes an enhanced mutation rate to higher levels of streptomycin resistance is in fact an R factor (6)

This paper reports the isolation of R factors and the distribution of drug resistance determinants in enteric bacteria from clinical sources

MATERIALS AND METHODS

Bacterial strains Strains of *Escherichia coli Klebsiella aerogenes* and *Proteus* were isolated in the diagnostic laboratory of this institute They were all recovered from in patients and represent strains thought to be of clinical significance Only strains were selected which exhibited resistance to at least three antibacterial drugs The drug sensitivity was determined by the paper disk method (5) The strains were also examined for prototrophy expressed as the ability to grow on a minimal medium

As recipients in the conjugation experiments were used auxotrophic strains of *E coli* K 12 originally supplied to this laboratory by H P Treffers Yale University T 71 met T 71 arg pro nal r T 71 his thr T 71 str r

Media Heart Infusion Broth agar (Difco) (HIB) was used as solid complete medium supplied with 0 5 per cent glucose The minimal medium was that described by Davis & Mingioli (4) with 0 5 per cent glucose added as carbon and energy source For selection purposes 10 μg/ml of the appropriate amino acids were incorporated Penassay Broth (Difco) was used as liquid medium in the conjugation experiments

Drugs The following drugs were used Chloramphenicol (Roussel Uclas Paris) tetracycline (Pharmaceutical Works Polsa Poland) streptomycin (Glaxo) ampicillin (Doctacillin Astra) sulphonamide (Sulphame athine ICI) and nalidixic acid (Winthrop)

Assay of drug resistance The average drug resistance was expressed as the maximal concentration of drug allowing full growth after streaking on plates incorporated with different concentrations of drugs For the determination of resistance to sulphonamide minimal medium was employed otherwise the medium was HIB agar

Culture method and conjugation procedure The multiple resistant donor strain and the recipient were grown in Penassay broth with gentle shaking to the logarithmic phase of growth Equal volumes were mixed and incubated stationary overnight at 37 C Samples were streaked on selective plates of HIB containing 9 μg/ml of chloramphenicol or 50 μg/ml of tetracycline and 50 μg/ml of nalidixic acid This medium prevented the growth of the donor cells but allowed the growth of the recipient T 71 arg pro nal r with transferred resistance to chloramphenicol or tetracycline

In those cases where the donor strain was resistant to nalidixic acid the recipient used was T 71 str r its streptomycin resistance thought to be of the chromosomal one step mutation type 1000 μg ml of streptomycin was incorporated in the selective medium The plates were incubated for 24 to 48 hours Several colonies were picked from each plate and streaked on the same medium for purification

The cl nes were then investigated for non selected markers by the paper disk sensitivity te t and by streaking on plates containing 10 μg/ml of streptomycin 50 μg/ml cm amp icillin and 200 μg/ml of sulphonamids The nutrition requirements of the recipient were also checked

Ability t further transfer of the transmitted R fact r was determined by con jugati n with T 71 met or T 71 his thr The drug resistant recipients were selecte l on minimal medium containing the amino acids require l by the recipient in ques ti n Before streaking the conjugati n mixture was washed twice in liqui l minimal medium without nutrients

Acri line treatment Rem val of the R factors was accomplished by growing the cells in 5 50 μg/ml of acriflavine in broth with shaking for 18 20 hours (15) Sur viving cells were plated on HIB plates without drugs and replicated onto drug c ntaining plates using the technique of Le lerberg (11) f r detection of clones with loss of resistance

RESULTS

A tot il of 147 str uns resistant to multiple drugs were ex imined in cluding 92 *E coli* 43 *K aerogenes* and 12 *Proteus* strains Table 1 shows the distribution of the str uns in isolates from clinical material 83 per cent were recovered from urinary tract infections in which the number of bacteria exceeded 10 per ml urine estimated with a quan titative technique (8)

The frequency of R factors is recorded in Table 2 and their patterns of resist ince determin ints are presented in Table 3 Most of the strains tr insferred all the resistance traits of the wild type Determinants for resistance to sulphonamide (Su) streptomycin (Sm) and chloramphe

TABLE 1

Distribution of Multiple Drug Resistant Strains of Enteric Bacteria in Isolates from Clinical Sources

	Urine	nose and throat swabs	Sputum	Pus	Total
E. coli	73	15	0	4	92
K aerogenes	38	2	1	2	43
Proteus	12	0	0	0	12
Total	123	17	1	6	147

TABLE 2

Frequency of R factors in Multiple Drug Resistant Strains of Escherichia coli Klebsiella aerogenes *and Proteus*

Organism	No of strains investigated	Strains carrying R factors no	per cent
E. coli	92	35	38
K aerogenes	43	31	72
Proteus	12	1	8
Total	147	67	45 5

TABLE 3

Pattern of Resistance Determinants in R factors Isolated from Escherichia coli *Klebsiella aerogenes and* Proteus

Multiple resistant wild type organism	Resistance determinants of R factors transferred	No of isolates
E coli	Su Sm Cm Tc Am	11
	Su Sm Cm Tc	17
	Su Sm Cm	6
	Su Sm Tc	1
K aerogenes	Su Sm Cm Tc Am	16
	Su Sm Cm Tc	11
	Su Sm Cm	3
	Sm Cm Tc Am	1
Proteus	Su Sm Cm	1

TABLE 4

Average Drug Resistance Level of E coli K12 T71 Carrying Various R factors Determined on Heart Infusion Broth Agar (µg/ml)

Strain	R factor	Cm	Sm	Tc	Am	Su
T71 his thr	R1(Su Sm Cm Am)	>500	>15 <25	< 10	>500	>1000
T71 arg pro nal r	R2(Su Sm Cm Tc)	>500	>10 <15	>100 <250	< 10	>1000
T71 arg pro nal r	R4(Su Sm Cm)	>400 <500	>15 <25	< 10	< 10	>1000
T71 arg pro nal r	R7(Su Sm Cm Tc Am)	>400 <500	15	>100 <250	>500	>1000
T71 arg pro nal r	(control)	< 10	< 5	< 10	< 10	< 50

Drug resistance to sulphonamides was determined on minimal medium agar plates.

Symbols used nal r nalidixic acid resistance his histidin arg arginin thr threonin pro prolin Cm chloramphenicol, Sm streptomycin Tc tetracycline Am ampicillin Su sulphonamide

nicol (Cm) almost invariably were transferred jointly The transfer of tetracycline (Tc) and ampicillin (Am) resistance failed in some few instances although the donor strain was resistant to these drugs In some cases it could be demonstrated that the reason for this was segregation of the resistance traits Alternatively the reason may be that these genes were located on the chromosome and not on the plasmid

The level of resistance was determined in the K12 system Corresponding values were obtained by testing different R factors The representative concentrations of antimicrobial drugs for the R recipient and four different R factors are shown in Table 4

Additional experiments were done in which the recipient strain was incubated with culture filtrate of multiple resistant donor strains known to be harbouring R factors *No transfer of resistance* was obtained This

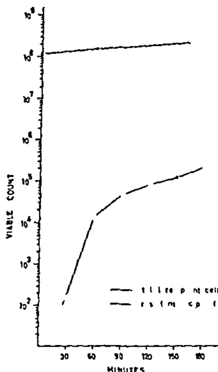


Fig 1

Kinetics of the transfer of an R factor in the K 12 system Donor T 71 his thr R8 (Su Sm Cm Tc) Recipient T 71 arg pro nal r The cultures were grown in Pen assay Broth to the logarithmic growth phase and mixed in equal volumes Aliquots were taken at intervals vigorously agitated for sixty seconds in a Vortex Jr Mixer diluted a thousandfold in Penassay Broth to prevent further conjugation and plated on Heart Infusion Broth agar plates containing 50 μg/ml of nalidixic acid and 25 μg/ml of chloramphenicol. Aliquots were also plated on plates supplemented with nalidixic acid only for the assay of the total recipient cells

TABLE 5

Loss of R factors in Strains of E. coli and One Strain of Salmonella typhimurium *Following Treatment with Acridine Dyes*

Strain	R factor	Acri flavine μg/ml	No of colonies scored	No of colonies with loss of R factor	Per cent
E. coli 2069	R3(Su Sm Cm Tc Am)	15	112	99	88 4
E. coli 11739	R4(Su Sm Cm)	40	700	0	0
S typhimurium HNI	R1(Su Sm Cm Am)	10	373	46	12 3
		20	315	59	18 7
K12 T71 his thr	R1(Su Sm Cm Am)	20	710	3	0 4
K12 T71 his thr	R8(Su Sm Cm Tc)	30 (3 sub cultures)	>1000	0	0

The resistant strain was grown overnight in Penassay broth with acriflavine Surviving cells were plated on HIB agar plates and replicated onto plates containing 25 μg/ml of chloramphenicol Clones with loss of chloramphenicol resistance were further investigated for loss of other determinants

indicates that the cell to cell contact of the conjugation process is necessary for the transfer and makes it highly unlikely that transduction by a phage is the mechanism of resistance transfer Interruption of the conjugation by seperating the conjugating cell pairs thus resulted in

interruption of the transfer as well The kinetics of transfer is shown in Fig 1

The wild type strains differed in their response to treatment with acriflavine (Table 5) A high rate of loss was found in the wild type strain *E coli* 2069 In a strain of *Salmonella typhimurium* HNI RI which is included for comparison there was a great loss even on one single subculture in broth without acriflavine Other strains were highly resistant to acriflavine in spite of their ready transmission of the R factor by conjugation K12 T71 thus exhibited a high grade of stability

DISCUSSION

This investigation indicates that multiple drug resistance in enteric bacteria isolated from clinical material in man very often is caused by resistance factors belonging to the bacterial plasmids

There is however reason to believe that the present results represent a minimal estimate and that the real frequency of R factors is even higher For one thing the ability of DNA transfer may not always be expressed or the frequency of transfer may be very low For unknown reasons bacterial strains may vary considerably in their compatibility This is true for strains belonging to different genera but also for strains of the same species

Seperate loss of the resistance transfer factor RTF may lead to a defective R factor incapable of conjugational transfer (2) This loss will not influenze the drug resistance of the cell The subsequent acquisition of the detached infective RTF may again mobilize the resistance determinants of the R factor (1)

There is evidence that R factors may determine the production of a cytoplasmic repressor repressing their own conjugating function (12) This is shown by the fact that bacteria with a newly acquired R factor will transmit it with high frequency This is known as high frequency resistance transfer (HFRT) (21) In a recipient with no preformed repressor the ability of transfer will be practically freely expressed for some few generations until the repressor appears in the cytoplasm In established strains the conjugating function may be completely repressed Mutant derepressed R factors with highly increased frequency of transfer have been isolated (13)

These facts indicate that the failure to demonstrate the conjugational transfer of drug resistance does not exclude the existence of plasmidal elements as the cause of resistance Thus the frequency of transfer was much lower in the strains of *E coli* which had been subcultured many times kept for a long time on agar slants or had been lyophilized than in the strains which were tested for transmissibility just after isolation

In this examination attention has been focused on strains resistant to three drugs or more According to most reports these are most fre

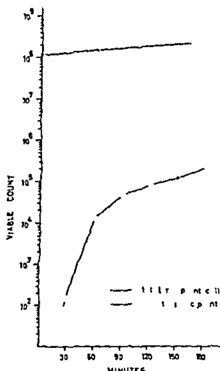


Fig 1
Kinetics of the transfer of an R factor in the K12 system Donor T71 his thr R8 (Su Sm Cm Tc) Recipient T71 arg pro nal r The cultures were grown in Pen assay Broth to the logarithmic growth phase and mixed in equal volumes Aliquots were taken at intervals vigorously agitated for sixty seconds in a Vortex Jr Mixer diluted a thousandfold in Penassay Broth to prevent further conjugation and plated on Heart Infusion Broth agar plates containing 50 µg/ml of nalidixic acid and 25 µg/ml of chloramphenicol Aliquots were also plated on plates supplemented with nalidixic acid only for the assay of the total recipient cells

TABLE 5
Loss of R factors in Strains of E. coli and One Strain of Salmonella typhimurium *Following Treatment with Acridine Dyes*

Strain	R factor	Acriflavine µg/ml	No of colonies scored	No of colonies with loss of *R factor*	Per cent
E coli 2069	R3(Su Sm Cm Tc Am)	15	112	99	88 4
E. coli 11739	R4(Su Sm Cm)	40	700	0	0
S typhimurium HNI	R1(Su Sm Cm Am)	10	373	46	12 3
		20	315	59	18 7
K12 T71 his thr	R1(Su Sm Cm Am)	20	710	3	0 4
K12 T71 his thr	R8(Su Sm Cm Tc)	30 (3 sub cultures)	>1000	0	0

The resistant strain was grown overnight in Penassay broth with acriflavine Surviving cells were plated on HIB agar plates and replicated onto plates containing 25 µg/ml of chloramphenicol Clones with loss of chloramphenicol resistance were further investigated for loss of other determinants

indicates that the cell to cell contact of the conjugation process is necessary for the transfer and makes it highly unlikely that transduction by a phage is the mechanism of resistance transfer Interruption of the conjugation by seperating the conjugating cell pairs thus resulted in

creted *E coli* with multiple resistance whereas 58 9 per cent of inpatients treated with chloramphenicol carried such strains

Given optimal conditions the transfer starts within few minutes (20) Fig 1 shows that most of the transfer has taken place within 60 min The *in vivo* transfer i e in the alimentary tract is probably less efficient There is reason to believe that without the disturbing effect on the ecological balance of drug administration the natural environment will not favour the spread of infectiously resistant strains The high rate of spontaneous loss is probably not less *in vivo* Mating resistant donor strains which also harbour transferable colicinogenic factors may be lethal for the noncolicinogenic recipient bacteria Introduction of this factor is then followed by colicin synthesis whereas this synthesis is repressed in the donor cells These facts together with the variation in compatibility and the effect of environmental conditions represent a negative selection of resistant strains The use of antibacterial drugs may easily counteract this and the existence of R factors in the normal intestinal flora may then involve a potential danger of transfer of multiple resistance to invading primarily sensitive strains of *Shigella* and *Salmonella*

In this country infection with coliform and Proteus organisms is the most common problem created by the enteric bacteria In particular infections of the urinary tract may be a serious therapeutic problem 83 per cent of the present strains were isolated from such infections most of these were cases of chronic pyelonephritis hydronephrosis paresis of the urinary bladder kidney transplantations and other lesions of the urinary tract A change of the bacterial flora during drug administration with the establishment of a strain with multiple resistance has frequently been observed There is no doubt that many of these infections are in fact crossinfections and superinfections The pattern of resistance determinants of the R factors shows a similarity which may reflect the fact that an originally limited number of R factors have spread in the hospital departments Infectious drug resistance may then be considered in the same way as the penicillinase resistant *Staph aureus* and the R factors certainly play a not insignificant role in the problem of nosocomial infections

SUMMARY

147 different strains of *E coli* *K aerogenes* and *Proteus* resistant to three or more antibacterial drugs have been examined for transfer factors 45 5 per cent of the strains were found to harbour *R* factors conveying resistance to three or more of the following drugs sulphonamide streptomycin tetracycline chloramphenicol and ampicillin The R factors were transferable by conjugation and were removed by treatment with acriflavine although more or less efficiently 83 per cent of the multiply resistant strains were recovered from urinary tract

infections most of them chronic infections combined with lesions of the urinary tract

The results of the investigations indicate that R factors play a major role in the multiple resistance of enteric bacteria causing infections in hospitals Such strains are undoubtedly responsible for many of the crossinfections and superinfections which constitute the problem of nosocomial infections

REFERENCES

1 *Anderson E S* A rapid screening test for transfer factors in drug sensitive Enterobacteriaceae Nature *208* 1016–1017 1965
2 *Anderson E S & Lewis M J* Characterization of a transfer factor associated with drug resistance in *Salmonella typhimurium* Nature *208* 843–849 1965
3 *Datta N* Infectious drug resistance Brit med Bull *21* 254–259 1965
4 *Davis B D & Mingioli E S* Mutants of *Escherichia coli* requiring methionine or vitamin B_{12} J Bact *60* 17–28 1950
5 *Ericsson H Högman C & Wickman K* A paper disk method for determination of bacterial sensitivity to chemotherapeutic and antibiotic agents Scand J Clin & Lab Invest Suppl 11 *6* 21–36 1954
6 *Gundersen W B* On the nature of the Mu factor Acta path microbiol scand *73* 93 105 1968
7 *Gundersen W B Jyssum K & Lie S* Genetic instability with episome mediated transfer in *Escherichia coli* J Bact *83* 616–623 1962
8 *Hoeprich P D* Culture of the urine J Lab clin Med *56* 899–907 1960
9 *Jacob F Schaffer P & Wollman E L* Episomic elements in bacteria Symposium Soc Gen Microbiol *10* 67–91 1960
10 *Lederberg J* Cell genetics and hereditary symbiosis Physiol Rev *32* 403–430 1952
11 *Lederberg J & Lederberg E M* Replica plating and indirect selection of bacterial mutants J Bact *63* 399–406 1952
12 *Meynell E & Datta N* The relation of resistance transfer factors to the F factor (sex factor) of *Escherichia coli K 12* Genet Res Camb *7* 134–140 1966
13 *Meynell E & Datta N* Mutant drug resistant factors of high transmissibility Nature *214* 885–887 1967
14 *Mitsuhashi S* Transmissible drug resistance factor R Gunma J med Sci *14* 169–209 1965
15 *Mitsuhashi S Harada K & Kameda M* Elimination of transmissible drug resistance by treatment with acriflavine Nature *183* 947 1961
16 *Mitsuhashi S Hashimoto H Egawa R Tanaka T & Nagai Y* Drug resistance of enteric bacteria IX Distribution of R factors in gram negative bacteria from clinical sources J Bact *93* 1242 1245 1967
17 *Smith D H & Armour S E* Transferable R factors in enteric bacteria causing infection of the genitourinary tract Lancet *II* 15–18 1966
18 *Smith H W & Halls S* Observations on infective drug resistance in Britain Brit med J *I* 266–269 1966
19 *Walton J R* Infectious drug resistance in *Escherichia coli* isolated from healthy farm animals Lancet *II* 1300–1302 1966
20 *Watanabe T* Infective heredity of multiple drug resistance in bacteria Bact Rev 27 87–115 1963
21 *Watanabe T* Episome mediated transfer of drug resistance in Enterobacteriaceae VI High frequency resistance transfer system in *Escherichia coli* J Bact *85* 788–794 1963
22 *Watanabe T & Lyang K W* Episome mediated transfer of drug resistance in Enterobacteriaceae V Spontaneous segregation and recombination of resistance factors in *Salmonella typhimurium* J Bact *84* 422–430 1962

Acta path microbiol scandinav 74 35–40 1968

Department of Toxoplasmosis and Viral Diseases (Head J Chr Siim M D)
Statens Seruminstitut Copenhagen Denmark

PREPARATION OF *TOXOPLASMA GONDII* ANTIGEN FOR THE COMPLEMENT FIXATION TEST

By

EILIV K PETTERSEN

Received 30 i 68

With a view to the extraction of a soluble complement fixing antigen from Toxoplasma gondii the first method to be described was that of repeated freezing and thawing of the parasites in physiological salt solution (*Warren & Sabin* 1942) This method of extraction has been adopted in most laboratories It has however been pointed out that repeated freezing and thawing reduced the titre of the antigen (*Kass* 1953) Furthermore the extraction procedure has been found to be somewhat timeconsuming and the present investigation was therefore undertaken in order to find a more suitable method for the extraction of the antigen A preliminary investigation has been reported (*Pettersen* 1967)

MATERIALS AND METHODS

Peritoneal exudate was collected from four week old white mice which had been infected intraperitoneally five days previously with 20 000 Toxoplasma gondii parasites of the BH strain (The mice used were of the Institute s own breed weighing 18–20 g at the time of infection) The exudate was centrifuged at 500 × g for 40 minutes The sediment containing the parasites together with a vast number of mouse cells was washed once with 0 15 M sodium chloride solution and stored at −18 C The average sediment contained 3×10^9 parasites and this constituted the least amount used for any of the extractions described below

Measured amounts of 0 15 M sodium chloride solution were added to the parasite sediments and the mixtures were treated briefly in a Potter Elvehjem tissue grinder

1 *Freezing and thawing* The parasite suspension was subjected three times to alternate freezing in a solid carbon dioxide ethanol mixture and thawing in water of 20–30 C followed by centrifugation Consecutive extractions were performed on each batch of parasite suspension

2 *Bicarbonate extraction* To the parasite suspension were added 25 2 mg of sodium bicarbonate per ml The suspension was stirred for 10 minutes and then diluted with two volumes of demineralized water The bicarbonate was neutralized by the careful addition of N HCl and the suspension was centrifuged The sediment was subjected to a second bicarbonate extraction

In part aided by grants to the Department of Toxoplasmosis and Viral Diseases from *King Christian X Foundation* and the *US Public Health Service Research Grant Foundation* (AI 1741) Bethesda Md.

The skilled technical assistance of Miss *E Lauritsen* is gratefully acknowledged

3 *Carbonate extraction* To the parasite suspension were added 16 mg of sodium carbonate per ml *The suspension was stirred for 10 minutes and then diluted with* one volume of demineralized water The carbonate was neutralized by the careful addition of N HCl and the suspension was centrifuged

Some of the alkaline extractions were *performed at room temperature and some* were performed as far as possible at 0–4 C. The centrifugation of the extracts was performed at 2–4 C using 34 000 × g for the periods necessary to obtain clear supernatants which were stored in *small portions at* –18 C *Consecutive antigen extracts* from the same batch of parasite *suspension were designated by* capital letters

The complement fixation test To a glass tube containing 0 03 ml of antigen solution and 0 075 of antiserum in their appropriate dilutions was added 0 15 ml of complement solution The tube was left for 45 minutes at room temperature and then *placed for 45 minutes on a water bath of 37* C. At this point 0 2 ml of a 2 5 per cent suspension of sheep red cells containing three amboceptor units was added and the tube was returned to the water bath After a further 45 minutes at 37 C the tube was removed and left at 4 C. The degree of haemolysis was determined the next *morning* taking 50 per cent haemolysis or less by visual observation as a positive result.

As complement was used guinea pig serum in a dilution corresponding to 0 75 haemolytic unit as determined spectrophotometrically at 520 nm

The antiserum *used throughout this study* was a human antitoxoplasma serum which was routinely tested for anticomplementary effect

Titration procedure Box titrations *were performed on the antigen extracts with* the above mentioned antiserum Four to six of these antigens were always titrated in parallel and at least one of them had been titrated before

All the antigens were tested for anticomplementary effect using as above 0 75 haemolytic unit of complement

Antigens from all the procedures mentioned were tested *for unspecific binding effect with a human serum found negative in the dye test and the complement* fixation test The antigens were also tested for lysing effect on the sheep red cell suspension

RESULTS

The results given in the Tables 1 2 3 and 4 are obtained from the extractions of pooled parasite sediments homogenized in 0 15 M sodium chloride solution The homogenous suspensions were in each case divided into two parts of which one half volume was used for the extraction by freezing and thawing (I) and the other half volume was used for the extraction with bicarbonate (II)

TABLE 1

Extraction from 15 × 10⁹ parasites in 45 ml

Antigen extract	Final antigen volume in ml *per 10⁹ parasites*	Antigen titre	Antiserum titre
I A	3	128	256
I B	3	32	256
I C	3	16	256
II A	9	64	1024
II B	9	16	256

I = *extract from freezing and thawing* II = bicarbonate extract
Capital letters designate consecutive extracts

Tables 1 and 2 give the results from bicarbonate extraction (II) performed at 4 C whereas the results in Table 3 refer to bicarbonate ex

traction (II) at room temperature Table 4 gives the result from two extractions performed with carbonate (III) at room temperature

TABLE 2

Extraction from 9 × 10⁹ Parasites in 30 ml

Antigen extract	Final antigen volume in ml per 10^9 parasites	Antigen titre	Antiserum titre
I A	4	64	128
I B	4	16	256
I C	4	8	256
II A	12	64	1024
II B	12	32	512

I = extract from freezing and thawing II = bicarbonate extract
Capital letters designate consecutive extracts

TABLE 3

Extraction from 6 × 10⁹ Parasites in 30 ml

Antiserum titre	Final antigen volume in ml per 10^9 parasites	Antigen titre	Antigen extract
I A	5	64	64
I B	5	16	256
I C	5	8	256
II A	15	32	1024
II B	15	16	256

I = extract from freezing and thawing II = bicarbonate extract.
Capital letters designate consecutive extracts

TABLE 4

Carbonate Extractions

Antigen extract	Number of parasites	Extraction volume in ml	Final antigen volume in ml per 10^9 parasites	Antigen titre	Antiserum titre
III	3×10^9	15	20	64	1024
III	3×10^9	30	33	32	512

The titres given in these tables are the reciprocal values of the highest dilution of the antigen solutions giving a positive complement fixation test with the antiserum diluted 1 2 and the highest dilution of the antiserum giving a positive complement fixation test with the undiluted antigen solutions

In order to facilitate comparison of the results from the different extraction procedures the titres were extrapolated to the values corresponding to a yield of 10 ml of antigen solution per 10^9 parasites

These calculated values which are given in Table 5 make it possible to compare the strengths of the various antigen preparations

The average antigen titre of I compared with those of II and III indicates that more than half of the available antigen was destroyed by the freezing and thawing procedure The antigen titres of II indicate that there was no significant difference in the results of extraction at 4 C and at room temperature The average antiserum titre of I A as compared with II A and III indicates that the antigens obtained by alkaline extractions are more than 20 times as potent as that obtained by freezing and thawing

TABLE 5

Titres Extrapolated to a Final Antigen Volume of 10 ml per 10^9 Parasites

Antigen	Antigen titre	Antiserum titre	Average titre of antigen	Average titre of antiserum
I A	38	77		
	26	51	32	53
	32	32		
I B	10	77		
	6	102	8	102
	8	128		
I C	5	77		
	3	102	4	102
	4	128		
II A	58	922		
	77	1229	61	1229
	48	1536		
II B	14	230		
	38	614	26	410
	24	384		
III	128	2048	117	1869
	106	1690		

I = extract from freezing and thawing II = bicarbonate extract
III = carbonate extract Capital letters designate consecutive extracts

None of these antigen solutions had anticomplementary unspecific binding or lysing effects under the conditions described above though it was found that repeated extractions beyond the stated number sometimes gave antigens which were anticomplementary

The antigen solutions could be lyophilized in their physiological salt solutions without any apparent loss of activity Storage of the antigens in physiological salt solution at —18 C reduced the titres on an average with less than 20 t. Some of these frozen antigen solutions were tested er st ar

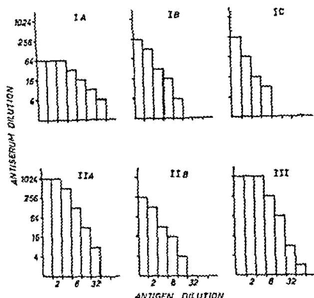


Fig 1

Box titration patterns of various antigen preparations
I = extract from freezing and thawing   II = bicarbonate extract
III = carbonate extract Capital letters designate consecutive extracts

DISCUSSION

The complement fixation test as performed in this study using two fold dilution steps is a rather crude analytical tool for the estimation of antigen potency Only four fold differences in titres would theoretically be significant for any two antigens With this in mind the extractions were deliberately varied in steps different from two with respect to extraction volume per unit number of parasites Furthermore average titres of three extracts were obtained by each procedure and the reproducibility of titrations has been ensured by including the titration of one or more reference antigens

As already pointed out the quotient of the antiserum by the antigen titre was considerably smaller for I A than for any of the other antigen extracts This is also apparent in Fig 1 illustrating the typical box titration patterns of the antigens in question A similar difference in box titration patterns has been demonstrated for the mouse peritoneal exudate antigen and an antigen from mouse embryo tissue culture (*Schuhova et al* 1961) The difference was considered due to the presence of an inhibitor in the mouse peritoneal exudate antigen

The inhibitor would be destroyed by alkaline treatment This was confirmed by treating an antigen solution I A with carbonate The carbonate treated solution and the untreated solution were diluted to the same extent and titrated in the complement fixation test The re

sults, given in Table 6 indicate that the potency of I A with respect to antiserum titre by the alkaline treatment was increased to the same level as that of the extracts II and III

TABLE 6
Alkaline Treatment of Antigen Solution I A

Antigen	*Antigen titre*	*Antiserum titre*
I A	32	32
I A treated with carbonate	32	512

CONCLUSION

For the preparation of the complement fixing antigen from Toxoplasma gondii the alkaline extraction method and the use of sodium carbonate in particular gave the reproducible high yields of specific antigen which makes it suitable as a standard method of preparation

SUMMARY

1 Alkaline extraction was shown to be a more efficient method for obtaining the complement fixing antigen than the method using freezing and thawing Of the methods tried the extraction with carbonate gave the highest yield of antigen

2 Alkaline extraction was also a more convenient method to use as it required attention only for about one hour

3 The inhibitor present in the extract obtained by freezing and thawing was destroyed by the alkaline treatment thus giving a more potent antigen

REFERENCES

1 *Adss Frik* Undersøkelser over Toxoplasma og Toxoplasmose Akademisk Trykningssentral Oslo 1954 p 93
2 *Pettersen Eilif K* Preparation of Toxoplasma gondii antigen for the complement fixation test Acta path microbiol scandinav Suppl *187* 87 1967
3 *Schuhova Vera Zaradood H C Slumpa G* Complement fixation Toxoplasma antigen prepared in tissue culture Proc first int Congr Protozool Prague 1961 p 371-378
4 *Warren Joel & Sabin Albert B* The complement fixation reaction in toxoplasmic infection Proc Soc exp Biol Med *51* 11-14 1942

Acta path microbiol scandinav 74 41–50 1968

The Institute of Medical Microbiology University of Uppsala Uppsala Sweden
and Department of Pediatrics Akademiska Sjukhuset Uppsala Sweden

THE *IN VITRO* EFFECT OF ASCORBIC ACID ON THE BACTERIAL GROWTH IN URINE

By

HÅKAN GNARPE[1] MAGNUS MICHAELSSON and STEN DREBORG

Received 5 II 68

The growth of a number of pathogenic bacteria is reduced in acid media The lowering of the urinary pH to bacteriostatic levels has therefore been used as an aid in the treatment of urinary tract infections (*Rotoski* 1898 *Clark* 1931 *Helmholz* 1931 *Leaw* 1940) Different acidifying agents such as mandelic acid beta hydroxy butyric acid (*Helmholz* 1937) and acetic acid (*Marshall* 1950) have been tried Although *Kass* has reported methionine to be effective (1957) other authors have been unable to confirm these results (*McDonald & Murphy* 1959)

In 1959 *McDonald & Murphy* suggested the use of ascorbic acid as an acidifying agent because of its high excretion in urine (*Smith* 1951) They found that a daily dose of 2 5 *g of* ascorbic acid lowered the urinary pH to levels below 5 5 Some authors however considered the doses used by *McDonald & Murphy* too small and suggested a dosage of 8 g per square meter of body surface daily in order to obtain an acidifying effect (*Travis Dodge Mink & Assemi* 1965)

Since other reducing substances such as gluthathion and cystein have no antibacterial activity (*Ericsson & Lundbeck* 1955) nonspecific reduction could *not* be the mechanism for suppression of bacterial growth The bactericidal effect has been ascribed to the formation of free hydroxyl radicals in the presence of cupric ions (*Grab* 1965)

As the mechanism of the antibacterial action of ascorbic acid still remains unclear this investigation was initiated in order to determine 1) the effect of ascorbic acid on the growth curve *of* the most common pathogenic bacteria of the urinary tract 2) the effect of the bacteria on the concentration of ascorbic acid in urine and 3) the effect of ascorbic acid on the bacterial growth curve at constant pH

The authors wish to thank *AB Pharmacia* Uppsala and Mrs *B Hagqvist* for valuable help with the determinations of ascorbic acid. The authors also wish to thank Assoc I *I Fdebo* for valuable discussion and criticism

[1] Present address The Institute of Medical Microbiology University of Uppsala Uppsala Sweden

MATERIALS AND METHODS

Bacterial strains The following test organisms were used in the first series of experiments *Proteus vulgaris* (the X 19 strain) In the second series *Proteus vulgaris Escherichia coli Alcaligenes faecalis Pseudomonas aeruginosa Staphylococcus albus* and *Streptococcus faecalis* and in the third series of experiments *Proteus vulgaris* and *Escherichia coli*

Bacterial growth The bacteria were grown in nutrient broth for 18 hours and were thereafter added to the urine in final concentrations between 10^3 and 2×10 cells per ml In the first and second experimental series each urine sample amounted to 150 ml while in the third series to 500 ml The bacteria were grown in Erlenmeyer flasks with sidetubes on a rotary shaker at 37 C. The bacterial growth was followed by determinations of viable counts Viable counts were made by plating 0 1 ml amounts on solid media after serial dilutions in saline The following media were used for *Proteus vulgaris* – desoxycholate agar (Difco) for *E coli* – endo agar (Difco) and for all the other organisms – nutrient agar (Difco)

Culture media Sterile pooled human urine which was free from bacteriostatic and bactericidal activity was used in the experiments Various amounts of l ascorbic acid (Merck AC) were dissolved in the urine in concentrations up to 2 4 mg per ml Subsequently the urine was placed at +4 over night The controls were grown in urine without ascorbic acid added

Experiments Five types of experiments were run

1) In the first series of experiments the ascorbic acid concentration was determined polarographically
2) In the second series the pH was followed but not adjusted No determinations of the ascorbic acid concentrations were made
3) In the third series of experiments the pH was continuously adjusted to 6 0 The ascorbic acid concentrations were determined titrimetrically
4) In the fourth experimental series ascorbic acid was added at 3 C and 13 hours in order to keep the concentration of ascorbic acid on a high level
5) In the fifth series of experiments the ascorbic concentrations were determined in sterile urine

All experiments were repeated at least twice All the graphs presented in this communication represent one typical experiment

pH measurements A titrator (Type TTT1 Radiometer Copenhagen) adjusted for pH measurement was used in the first and second series of experiments In the third and fourth series of experiments in which the pH was continuously adjusted to 6 0 the titrator was adjusted for downward titration using 90 per cent lactic acid (not exceeding 5 ml per 500 ml of urine) for the titration

Determinations of ascorbic acid Samples were taken at various intervals The bacteria were spun down and the supernatant was drawn off and frozen at —20 C. In the first series of experiments the ascorbic acid concentration was determined polarographically according to *Brezina & Zuman* (1958) while in the third fourth and fifth experimental series this concentration was determined titrimetrically according to *Harris & Ray* (1935)

RESULTS

The Influence of Varying Concentrations of Ascorbic Acid on the Bacterial Growth

1) *First experimental series* When Proteus vulgaris grew in urine it was found that the ascorbic acid concentration decreased from 0 4 to 0 1 mg per ml during 6 hours (Fig 1) During the same time the viable count was reduced from 2×10^7 to 2×1 per ml T creased from 6 50 to 7 75 After 12 h ic acid r The viable count then increased and reac of 10^9 cell after 24 hours In the control the viable l from

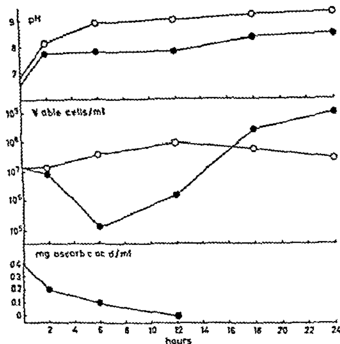


Fig 1

Growth of *P vulgaris* in urine with (—●—) and without (—○—) ascorbic acid added.

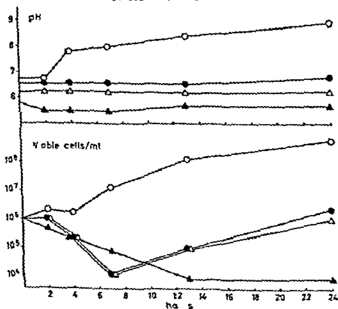


Fig 2

Influence of various concentrations of ascorbic acid on the growth of *P vulgaris* in urine ○ 0 mg/ml, ● 0.5 mg/ml △ 1.0 mg/ml ▲ 2.0 mg/ml

MATERIALS AND METHODS

Bacterial strains The following test organisms were used In the first series of experiments *Proteus vulgaris* (the X 19 strain) In the second series *Proteus vulgaris Escherichia coli Alcaligenes faecalis Pseudomonas aeruginosa Staphylococcus albus* and *Streptococcus faecalis* and in the third series of experiments *Proteus vulgaris* and *Escherichia coli*

Bacterial growth The bacteria were grown in nutrient broth for 18 hours and were thereafter added to the urine in final concentrations between 10^5 and 2×10 cells per ml In the first and second experimental series each urine sample amounted to 150 ml while in the third series to 500 ml The bacteria were grown in Erlenmeyer flasks with sidetubes on a rotary shaker at 37 C. The bacterial growth was followed by determinations of viable counts Viable counts were made by plating 0 1 ml amounts on solid media after serial dilutions in saline The following media were used for *Proteus vulgaris* – desoxycholate agar (Difco) for *E coli* – endo agar (Difco) and for all the other organisms – nutrient agar (Difco)

Culture media Sterile pooled human urine which was free from bacteriostatic and bactericidal activity was used in the experiments Various amounts of l ascorbic acid (Merck AG) were dissolved in the urine in concentrations up to 2 4 m per ml Subsequently the urine was placed at +4 over night The controls were grown in urine without ascorbic acid added

Experiments Five types of experiments were run

1) In the first series of experiments the ascorbic acid concentration was determined polarographically
2) In the second series the pH was followed but not adjusted No determinations of the ascorbic acid concentrations were made
3) In the third series of experiments the pH was continuously adjusted to 6 0 The ascorbic acid concentrations were determined titrimetrically
4) In the fourth experimental series ascorbic acid was added at 3 C and 13 hours in order to keep the concentration of ascorbic acid on a high level
5) In the fifth series of experiments the ascorbi concentrations were determined in sterile urine

All experiments were repeated at least twice All the graphs presented in this communication represent one typical experiment

pH measurements A titrator (Type TTT1 Radiometer Copenhagen) adjusted for pH measurement was used in the first and second series of experiments In the third and fourth series of experiments in which the pH was continuously adjusted to 6 0 the titrator was adjusted for downward titration using 20 per cent lactic acid (not exceeding 5 ml per 500 ml of urine) for the titration

Determinations of ascorbic acid Samples were taken at various intervals The bacteria were spun down and the supernatant was drawn off and frozen at —20 C In the first series of experiments the ascorbic acid concentration was determined polarographically according to *Brezina* & *Zuman* (1958) while in the third fourth and fifth experimental series this concentration was determined titrimetrically according to *Harris* & *Ray* (1935)

RESULTS

The Influence of Varying Concentrations of Ascorbic Acid on the Bacterial Growth

1) *First experimental series* When Proteus vulgaris grew in urine it was found that the ascorbic acid concentration decreased from 0 4 to 0 1 mg per ml during 6 hours (Fig 1) During the same time the viable count was reduced from 2×10^7 to 2×10^5 cells per ml The pH increased from 6 50 to 7 75 After 12 hours no ascorbic acid remained The viable count then increased and reached a level of 10^8 cells per ml after 24 hours In the control the viable count increased from 2×10^7

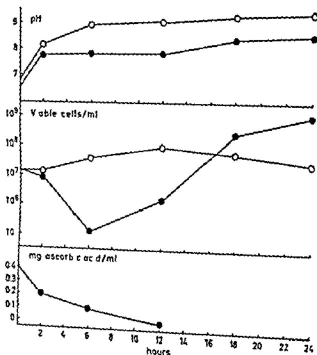


Fig 1
Growth of *P vulgaris* in urine with (—●—) and without (—○—) ascorbic acid added

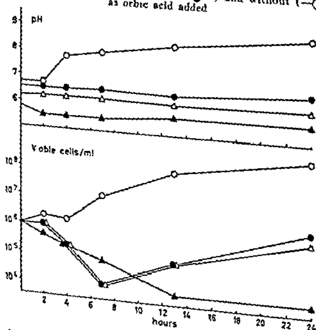


Fig 2
Influence of various concentrations of ascorbic acid on the growth of *P vulgaris* in urine ○ 0 mg/ml, ● 0 5 mg/ml △ 1 0 mg/ml ▲ 2 0 mg/ml

MATERIALS AND METHODS

Bacterial strains The following test organisms were used In the first series of experiments *Proteus vulgaris* (the X 19 strain) In the second series *Proteus vulgaris Escherichia coli Alcaligenes faecalis Pseudomonas aeruginosa Staphylococcus albus* and *Streptococcus faecalis* and in the third series of experiments *Proteus vulgaris* and *Escherichia coli*

Bacterial growth The bacteria were grown in nutrient broth for 18 hours and were thereafter added to the urine in final concentrations between 10^5 and 2×10 cells per ml In the first and second experimental series each urine sample amounted to 150 ml while in the third series to 500 ml The bacteria were grown in Erlenmeyer flasks with sidetubes on a rotary shaker at 37 C The bacterial growth was followed by determinations of viable counts Viable counts were made by plating 0 1 ml amounts on solid media after serial dilutions in saline The following media were used for *Proteus vulgaris* – desoxycholate agar (Difco) for *E coli* – endo agar (Difco) and for all the other organisms – nutrient agar (Difco)

Culture media Sterile pooled human urine which was free from bacteriostatic and bactericidal activity was used in the experiments Various amounts of ascorbic acid (Merck AC) were dissolved in the urine in concentrations up to 2 4 mg per ml Subsequently the urine was placed at +4 over night The controls were grown in urine without ascorbic acid added

Experiments Five types of experiments were run

1) In the first series of experiments the ascorbic acid concentration was determined polarographically
2) In the second series the pH was followed but not adjusted No determinations of the ascorbic acid concentrations were made
3) In the third series of experiments the pH was continuously adjusted to 6 0 The ascorbic acid concentrations were determined titrimetrically
4) In the fourth experimental series ascorbic acid was added at 3 6 and 13 hours in order to keep the concentration of ascorbic acid on a high level
5) In the fifth series of experiments the ascorbic concentrations were determined in sterile urine

All experiments were repeated at least twice All the graphs presented in this communication represent one typical experiment

pH measurements A titrator (Type TTT1 Radiometer Copenhagen) adjusted for pH measurement was used in the first and second series of experiments In the third and fourth series of experiments in which the pH was continuously adjusted to 6 0 the titrator was adjusted for downward titration using 20 per cent lactic acid (not exceeding 5 ml per 500 ml of urine) for the titration

Determinations of ascorbic acid Samples were taken at various intervals The bacteria were spun down and the supernatant was drawn off and frozen at –20 C In the first series of experiments the ascorbic acid concentration was determined polarographically according to *Brezina & Zuman* (1958) while in the third fourth and fifth experimental series this concentration was determined titrimetrically according to *Harris & Ray* (1935)

RESULTS

The Influence of Varying Concentrations of Ascorbic Acid on the Bacterial Growth

1) *First experimental series* When Proteus vulgaris grew in urine it was found that the ascorbic acid concentration decreased from 0 4 to 0 1 mg per ml during 6 hours (Fig 1) During the same time the viable count was reduced from 2×10^7 to 2×10^5 cells per ml The pH increased from 6 50 to 7 75 After 12 hours no ascorbic acid remained The viable count then increased and reached a level of 10^9 cells per ml after 24 hours In the control the viable count increased from 2×10^7

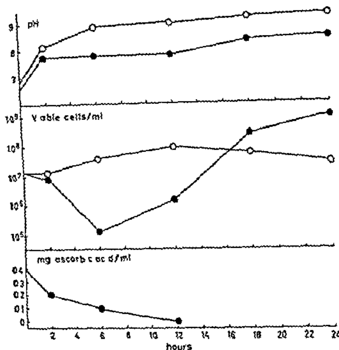


Fig 1

Growth of *P vulgaris* in urine with (—●—) and without (—○—) ascorbic acid added

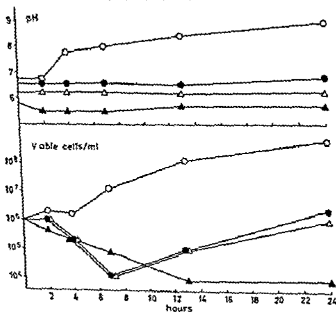


Fig 2

Influence of various concentrations of ascorbic acid on the growth of *P vulgaris* in urine ○ 0 mg/ml ● 0 5 mg/ml △ 1 0 mg/ml ▲ 2 0 mg/ml

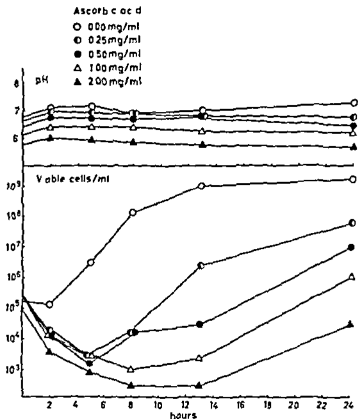


Fig 3

Influence of various concentrations of ascorbic acid on the growth of E coli in urine

to 6×10^7 cells per ml while the pH increased from 6 70 to 8 90 in 6 hours When the pH reached 9 0 no additional bacterial growth was observed

2) Second experimental series When overnight broth cultures of *P vulgaris E coli* and *Ps aeruginosa* were inoculated into urine with ascorbic acid added in concentrations up to 2 0 mg per ml the results are given in Figs 2 3 and 4 respectively No correction of the pH was undertaken during these experiments

Fig 2 shows that the viable count for the proteus bacteria was reduced from 10^5 to 9×10^3 cells per ml during 13 hours when ascorbic acid had been added to a concentration of 2 0 mg per ml The pH remained below 6 0 In the control without ascorbic acid the viable count increased from 10^6 to 2×10^9 cells per ml There was a rapid change of the pH from 6 75 to 8 40 during the same time

Fig 3 indicates that the viable count was reduced from 10^5 to 5×10 cells per ml with a slight change in the pH from 6 10 to 6 25 during the 13 hour period when *E coli* grew in urine with an ascorbic acid concentration of 2 0 mg per ml In the control the viable count increased from

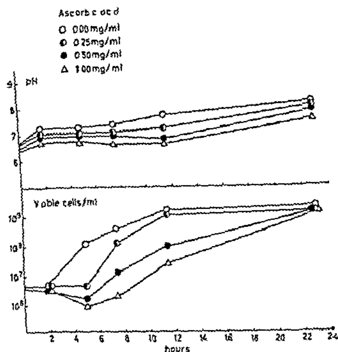


Fig 4

Influence of various concentrations of ascorbic acid on the growth of *Ps aeruginosa* in urine

3×10^6 to 10^9 cells per ml while the pH changed from 6 75 to 7 00 during the same time

Fig 4 shows for *Ps aeruginosa* grown under the same experimental conditions that the count increased from 6×10^6 to 5×10^7 cells per ml and the pH changed from 6 40 to 6 60 during 11 hours In the control the viable count increased from 7×10^6 to 3×10^9 cells per ml The pH increased from 6 70 to 7 70 during the same period of time

Alc faecalis Staph albus and *Str faecalis* behaved similarly to *E coli* i e the viable counts were markedly reduced when ascorbic acid was present

The Influence of Ascorbic Acid on the Bacterial Growth at Constant Hydrogen Ion Concentration

3) *Third experimental series* The result obtained when *P vulgaris* and *E coli* were grown in urine at constant pH (6 0) are given in Figs 5 and 6 respectively

When proteus bacteria grew in urine with ascorbic acid present the ascorbic acid concentration was reduced from 0 8 to 0 3 mg per ml during 9 hours During the same length of time the viable count was reduced from 10^7 to 10^5 cells per ml When the ascorbic acid concentra

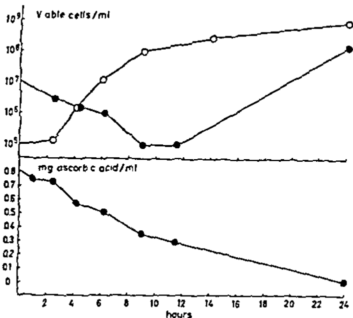


Fig 5
Growth of P vulgaris in urine with (—●—) and without (—○—) ascorbic acid added at pH 6 0

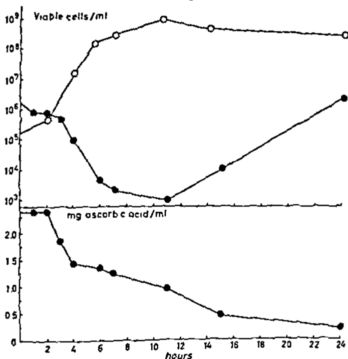


Fig 6
Growth of E coli in urine with (—●—) and without (—○—) ascorbic acid added at pH 6 0

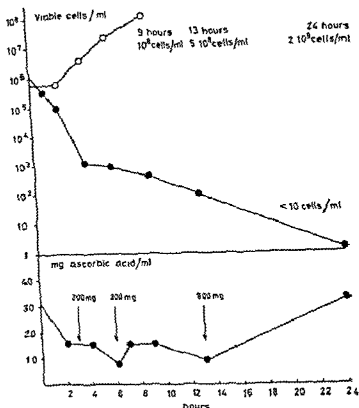


Fig 7

Growth of E coli in urine to which ascorbic acid was added at various intervals

tion was further reduced the viable count increaced to 2 × 10^8 cells per ml after 24 hours In the control the viable count increased from 10 to 10^8 cells per ml during 9 hours

When *E coli* grew under the same circumstances the ascorbic acid concentration was reduced from 2 4 to 0 95 mg per ml during 11 hours The viable count decreased from 3 × 10^4 to 10^3 cells per ml during the same time When the ascorbic acid concentration was further reduced the bacterial growth began again In the control the viable count increased from 3 × 10 to 10^5 cells per ml during the first 11 hours

4) *Fourth experimental series* When *E coli* grew in urine to which ascorbic acid was added at various intervals (*Fig 7*) the ascorbic acid concentration was maintained at a level over 0 8 mg per ml The viable count was reduced from 10^6 to 10 cells per ml in 13 hours and to below 10 viable organisms per ml in 24 hours In the control the viable count increased from 9 × 10^5 to 2 × 10^9 cells per ml during 24 hours

5) *Fifth experimental series* Under the same experimental conditions as in the first and second experimental series the concentration of a-

14 *Rotoski O* Ueber den bactericiden Einfluss der Acidität des Harns auf die Cystitiserreger Dt ch Med Wschr 24 235 236 and 249-252 1898
15 *Rustigian R & Stuart C A* The biochemical and serological relationship of the organisms of the genus Proteus J Bact 49 419 1945
16 *Smith H* In The Kidney Structure and Function in Health and Disease 1049 pp New York Oxford 1951 p 336
17 *Travis L B Dodge W F Mint A A & Assemi M* Urinary Acidification with Ascorbic Acid J Ped 67 1176-1178 1965
18 *Yeau R C* The Effect of pH on the Growth of Bacteria in Urine J Urol 44 699 713 1940

Acta path. microbiol. scandinav. 74: 51–60, 1968

The Biophysical and The Tuberculosis Department, Statens Seruminstitut, Copenhagen, Denmark

LOCALIZATION OF ACID PHOSPHATASE ACTIVITY IN MYCOBACTERIAL CELLS WITH THE ELECTRON MICROSCOPE

By

MORTEN H. NIELSEN and JØRGEN BENNEDSEN

Received 10.II.68

Certain enzymatic activities of the mycobacterial cell have been employed during recent years in the identification and classification of mycobacteria. Among the enzymes used are the acid phosphomonoesterases (acid phosphatases), since these are present only in certain species of mycobacteria (9, 10, 18).

It has been shown by others (1, 4, 16) that bacterial acid phosphatase as well as different neutral phosphatases and pyrophosphatases are confined to the superficial cell layers of gram negative bacteria, i.e. to the cell surface, the cell wall, or the space between the cytoplasmic membrane and the cell wall.

To our knowledge the question of where the acid phosphatases are localized in the mycobacterial cell has not as yet been answered. It is therefore the aim of the present study to determine where this enzyme activity of the mycobacterial cell is localized and thereby obtain information which would permit suggestions regarding the functional role of mycobacterial acid phosphatase.

MATERIAL AND METHODS

Four strains of *M. phlei* (Nos. 115, F 89, 29159, 18935), one strain of *M. kansasii* (N. TR 14) and five strains of *M. tuberculosis* (Nos. R 185, 6514, 3793, 4107, 11146) were examined. The strains were grown at 37° C on Löwenstein-Jensen solid medium and in Dubos liquid medium containing 0.05 per cent Tween 80.

The cells were harvested after 48 hours of growth for the rapidly growing and three to six weeks for the slowly growing strains.

The colonies on Löwenstein-Jensen medium were directly emulsified in the test tubes with a few drops of 1.5 per cent or 3 per cent glutaraldehyde in Michaelis buffer pH 6.3. The suspension was transferred to centrifuge tubes containing a few millilitres of glutaraldehyde fixative and then further emulsified with a glass rod. After fixation for 15 minutes the cells were washed twice in acetate buffer pH 5 by means of centrifugation. Cells cultivated in the Dubos medium were centrifuged, then fixed in glutaraldehyde and washed in acetate buffer as described for the cells grown on Löwenstein-Jensen medium. The pellet from the last centrifugation was suspended in 2 per cent agar at 40° C, and then slices or small blocks were prepared. In some experiments the cells were frozen and thawed repeatedly prior to suspension in agar.

The Gomori lead salt method as modified by *Holt* (5) was employed for the histochemical demonstration of acid phosphatase. In this method the enzymatic splitting of β glycerophosphate is indicated by precipitation of lead phosphate. The Gomori substrate contained 0.01 M sodium β glycerophosphate and 0.004 M lead nitrate in 0.05 M acetate buffer pH 5.0. In all experiments some of the blocks were incubated for the same period in two control substrates: (1) Gomori substrate with an enzyme poison (0.01 M sodium fluoride) and (2) Gomori substrate without β glycerophosphate.

The enzymatic reaction was observed by light microscopy after first adding diluted ammonium sulphide to blocks which were washed in diluted acetic acid. In order to obtain light microscopical evidence of enzyme activity, incubation for 1 to 4 hours at 37 C was required.

The specimens for electron microscopy were washed in dilute acetic acid after incubation in the Gomori and the control media and the treatment with dilute ammonium sulphide was omitted. Subsequently the blocks were fixed in osmium tetroxide (1 per cent in Michaelis buffer pH 6.1) for 18 hours. After osmium fixation the blocks were treated for one hour with 1 per cent buffered uranyl acetate pH 6.1 (14) and then dehydrated in alcohol and propylene oxide before embedding in Vestopal W (15). Ultrathin sections were cut by an LKB Ultrotome. Prior to electron microscopy the sections were double stained with magnesium uranyl acetate (3) and lead citrate (13). Electron micrographs were obtained with a Phillips EM 200 electron microscope.

RESULTS

Ultrastructure of the Mycobacterial Cell

With regard to electron density three layers can be distinguished in the cell wall of the mycobacteria *viz.* a wide inner electron dense layer (IL—Figs 1 3 4 8 9) a wide middle zone with no electron density (ML—Figs 3 4 9) and a very thin moderately dense outer layer (SL—Figs 1 3 4 8 9). As a rule the outer electron dense layer is more delicate in cells grown in Dubos medium, in a few cells it is almost invisible (SL—Figs 8 9).

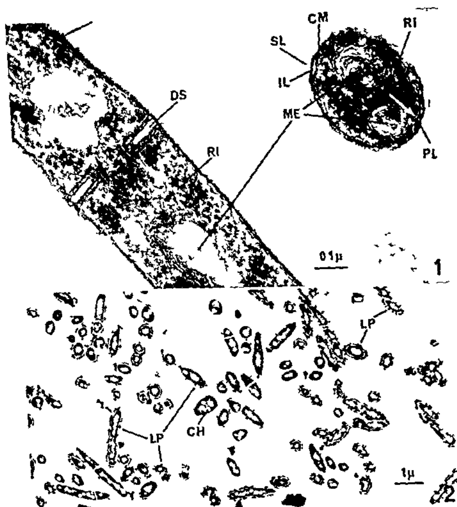
The cell wall and the cytoplasmic membrane are seperated by a distinct electron translucent layer in the *M phlei* strains (Arrow—Fig 3) but not in the *M tuberculosis* (Fig 8) or *M kansasii* strains (Fig 9). Occasionally this layer is bridged by small electron dense somewhat irregular cross bars (Arrow—Fig 5).

The cytoplasmic membrane is approximately 70 Å wide. It has the same morphological features in all the strains examined, the outer electron dense layer (PL—Figs 1 8 9) being wider and more electron dense than the inner layer (Arrow—Figs 1 8 9).

The nuclear region displays a substructure of delicate electron dense filaments in a matrix of low electron density (NU—Figs 3 4 8).

In addition to ribosomes (RI—Figs 1 3 4) large mesosomes (2) may be seen in the cytoplasm. These are usually located in the vicinity of the nuclear region (ME—Figs 1 3 4 8 9). Because of exposure to lead nitrate the polymetaphosphate granules within the cytoplasm often appear in the form of very electron dense inclusions (MG—Figs 5 6 7).

Certain areas in the cytoplasm show a very low electron density. Some have a circular circumference and are holes due either to evaporated metaphosphate granules (CH—Fig 8) or more probably to dissolved



Figs 1–9

Fig 1 *M phlei* (Strain 115) grown for 48 hours in Dubos medium Control experiment with cells incubated for 3 hours in Gomori substrate without β glycerophosphate added The outer layer (SL) of the cell wall is barely visible on one of the cells Between the inner electron dense layer of the cell wall (IL) and the cytoplasmic membrane (CM) there is an electron translucent layer The outer layer of the cytoplasmic membrane (PL) is more electron dense than the inner (arrow) The division septum (DS) is composed of the cytoplasmic membrane the inner electron dense layer and the electron translucent layer of the cell wall The mesosomes (ME) are not well preserved in the dividing cell Ribosomes denoted (RI) Magnification 87 000 ×

Fig 2 *M phlei* (Strain 115) grown for 48 hours in Dubos medium Cells incubated for 1 hour in Gomori substrate Lead phosphate precipitates (LP) are scattered over the cell surfaces The intensity of the histochemical reaction shows considerable variation from one cell to another (CH) denotes circular holes caused either by evaporated metaphosphate granules dissolved lipids or unstained granules of unknown composition Magnification 7 400 ×

01μ
RI
CL
LP
CM
IL
ML
SL
ME
NU
3
01μ
RI
CL
IL
ML
SL
ME
NU
4

lipids or unstained granules of unknown composition (CH—Figs 2 7) Others are of a more cloudy configuration (CL—Figs 3 4) Both types are generally displaced towards the cell poles

Histochemical Findings

Acid phosphatase activity was found in all the *M phlei* and the *M kansasii* strains but not in the *M tuberculosis* strains As regards the *M phlei* strain F 89 and the *M kansasii* strain the percentage of cells with acid phosphatase activity was low when the cells were grown in Dubos medium but clusters of cells with acid phosphatase activity could always be observed in the electron microscope (Fig 6) In these cases the light microscopical evaluation of the histochemical reaction was uncertain

The lead phosphate precipitates which are due to the enzymatic activity of acid phosphatase are confined to the outer layer of the cell wall and the space just outside the cell (LP—Figs 2 3 5 6 10)

The precipitates outside the cells usually appear in the form of elongated crystals radiating from the cell surface whereas those in the outer layer of the cell wall have a more amorphous configuration

Cells incubated in the two control media have no lead precipitates on the cell surface If there are precipitates these are confined to certain cytoplasmic granules only (MG—Fig 7)

DISCUSSION

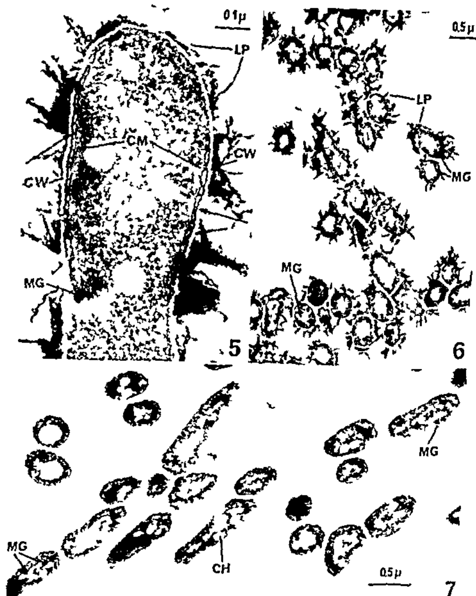
Our results concerning the ultrastructure of mycobacteria correlate with the findings reported from other laboratories (6 7 8 11 12) It would thus seem that the outstanding morphological feature of the mycobacterial cell is confined to the substructure of the cell wall This morphological finding may be correlated with the high lipid content of the cell wall as it has been found by chemical analysis (17)

We have observed that the cytoplasmic membrane and the cell wall of *M phlei* are seperated by an electron translucent layer whereas the

---

Figs 3 and 4

Cells of *M phlei* (Strain 115) grown for 48 hours on Lowenstein Jensen medium The cell in Fig 3 was incubated for 3 hours in Gomori substrate and that in Fig 4 in Gomori substrate with 0 01 M NaF added as enzyme poison Needle shaped and more amorphous lead phosphate precipitates (LP) are seen only on the cell in Fig 3 The amorphous precipitates are confined to the outer layer of the cell wall (SL) The nuclear areas (NU) with mesosomes (ME) are located centrally in the cell whereas other less electron dense areas in the cytoplasm (CL) are displaced toward the cell poles Ribosomes are marked (RI) Between the cytoplasmic membrane (CM) and the inner layer of the cell wall (IL) is an electron translucent space (arrow) The outer layer (SL) of the cell wall and the inner layer (IL) are separated by the electron translucent layer (ML) Magnification 87 000 ×



Figs 5–7

Figs 5 and 6 *M phlei* (Strain F 89) grown for 48 hours in Dubos medium Cells incubated in Gomori substrate for 3 hours The long crystal shaped lead phosphate precipitates (LP) probably indicate an overincubation in the substrate Even in this case there is no precipitation of lead phosphate in the interior of the cell The precipitates show scattered localization on the cell surfaces In the space between the cell wall (CW) and the cytoplasmic membrane (CM) small electron dense somewhat irregular cross bars can be seen (arrow Fig 5) Within the cytoplasm are small granules stained by the lead salts (MG) Magnification Fig 5 87 000 × Fig 6 14 800 ×

Fig 7 *M phlei* (Strain F 89) grown for 48 hours in Dubos medium Control experiment with cells incubated for 3 hours in Gomori substrate with 0 01 M NaF added as enzyme poison There are no lead phosphate precipitates on the cell

cytoplasmic membrane and the cell wall of *M tuberculosis* and *M kansasii* are in intimate contact

This difference between the rapidly and slowly growing strains is substantiated by the morphological features reported by other workers concerning *M phlei* (12) *Jucho* (8) *M lepra* and *M lepraemurium* (6 7)

Examination of a more representative number of strains is required before it can be established whether this difference between the rapidly and the slowly growing strains has a general validity

Kolbel (11) claims that the peripheral electron dense layer of the cell wall is not part of the cell wall proper but should be considered as an absorbed layer because of its special staining properties and in constant morphological features Both our histochemical and morphological results support the postulate that this peripheral layer is unique However we do not consider it to be merely an absorbed layer since at least part of it is formed by the cell and consists for instance of the surface enzymes demonstrated in the present study

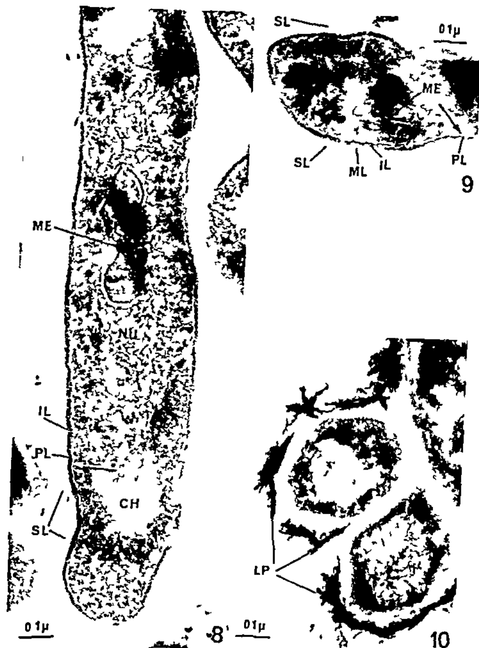
We were not able to demonstrate any acid phosphatase in the five *M tuberculosis* strains This corresponds to the findings of *Urabe et al* (18) In contrast Käppler (10) found acid phosphatase in all the *M tuberculosis* strains examined by him An explanation of this discrepancy may be that both *Urabe* and the writers employed β glycerophosphate as substrate whereas *Käppler* used colamin phenolphthalein phosphate Because all the β glycerophosphatase negative strains of *M tuberculosis* from our laboratory have a strong colamin phenol phtalein phosphatase activity as determined according to Käpplers method (unpublished observations) it is most likely that different enzymes are involved

The variations in the acid phosphatase activity found in *M phlei* cultures from the two media may be explained either as dependency on substrate or as an interaction of the Tween 80 in the Dubos medium Whatever the explanation this situation is a possible source of error in routine acid phosphatase determinations using different media

It may be concluded from the localization of the lead phosphate precipitates that the acid phosphatases are surface enzymes and that the enzymes conceivably are located in the outer electron dense layer of the mycobacterial cell wall We have not been able to demonstrate whether also the acid phosphatases of mycobacteria are exoenzymes nor have we demonstrated that enzyme activity is found towards the interior of

---

urfaces The electron dense granules (MC) within the cytoplasm are probabl metaph phate granules Circular h les (CH) in the cytoplasm are of unknown origin They may be due to extracted lipids evaporated metaphosphate granules or unstained granules of unknown composition Magnification 27 000 ×



Figs 8 10

Fig 8 *M tuberculosis* (Strain R 185) grown for 4 weeks in Dubos medium Cells incubated for 3 hours in Comori substrate with 0 01 M NaF added as enzyme poison The outer layer of the cell wall (SL) is barely visible whereas the inner layer (IL) is well developed The outer layer of the cytoplasmic membrane (I L) is more ele tron dense than the inner layer (arrow) The cyto plasmic membrane and the cell wall are in intimate contact Cl e to the nu clear area (NL) is a mes me (ME) At the cell pole is a hole (CH) proba bly due to an evaporated metaphosphate granule Magnification 87 000 ×

the cell However this may due to the experimental conditions employed For instance the cell wall may not have been opened sufficiently by the freezing/thawing procedure to permit an intracellular diffusion of the β glycerophosphate

The lead phosphate precipitates in most of the mycobacterial cells are confined to small areas of the cell surface It may be questioned whether this reflects a definite localization of the enzyme thus indicating a certain functional differentiation of the mycobacterial cell surface However conclusions concerning this interesting point cannot be based exclusively on this kind of histochemical observation Artefacts such as diffusion along the cell surface of newly formed but still not precipitated lead phosphates may confuse the issue

Since the acid phosphatases are hydrolases their location on the mycobacterial cell surface is compatible with functions involving hydrolysis of foreign high molecular substances This role would correspond to that proposed for acid phosphatases in gram negative bacteria (4) or in phagolysosomes of metazoan and protozoan cells

In more highly developed cells the acid phosphatases are also engaged in cell autolysis We do not know whether this also might be the case with mycobacteria since the cell populations examined in this study showed a negligible number of autolysing cells

SUMMARY

The Gomori lead salt technique for the histochemical demonstration of acid phosphatase was applied to three species of mycobacteria *viz* *M phlei* *M tuberculosis* and *M kansasii* The histochemical reaction was observed by electron microscopy of ultrathin cell sections

It was concluded that the acid phosphatase was a surface enzyme on cells of the *M phlei* and the *M kansasii* strains whereas no acid phosphatase activity was found on cells of *M tuberculosis*

For the strains with acid phosphatase activity the number of reacting cells varied in the different cell populations examined and the intensity of the staining reaction differed from one cell to another

---

Fig 9 *M kansasii* (Strain FR 14) grown on Lowenstein Jensen medium for 4 weeks Cells incubated in Gomori substrate for 3 hours As regards substructure this cell displays the same morphological features as the cell of *M tuberculosis* (Fig 7) Between the outer layer (SL) and the inner layer (IL) of the cell wall is the electron translucent middle layer (ML) The outer electron dense layer of the cytoplasmic membrane (PL) is in intimate contact with the inside of the cell wall (IL) The inner electron dense layer of the cell wall is more delicate than the outer layer (arrow) Part of a mesosome (MF) can be seen Magnification 87 000 ×

Fig 10 *M kansasii* (Strain FR 14) grown on Lowenstein-Jensen medium for 4 weeks Cells inculated for 3 hours in Gomori substrate The large needle shaped lead phosphate precipitates (LP) are confined to the cell surface Magnification 8 000 ×

The overall percentage of reacting cells was dependent to some extent on the medium used or whether the cells were cultivated on Dubos or on Lowenstein Jensen media

The ultrastructure of the three species of mycobacteria is described and the findings are discussed with special regard to the ultrastructure of the cell wall

REFERENCES

1 *Done J Shorey C D Loke J P & Pollak J K* The cytochemical localization of alcaline phosphatase in *E coli* at the electron microscope level Biochem J *96* 27C–28C 1965

2 *Fitz James P C* Participation of the cytoplasmic membrane in the growth and spore formation of bacilli J biophys biochem Cytol *8* 507 528 1960

3 *Frasca J M & Parks V R* A routine technique for double staining ultrathin sections using uranyl and lead salts J Cell Biol *25* 157 161 1965

4 *Heppel L A* Selective release of enzymes from bacteria Science *156* 1451–1455 1967

5 *Holt S J* Factors governing the validity of staining methods for enzymes and their bearing upon the Gomori acid phosphatase technique Exp Cell Res Suppl *7* 1–27 1959

6 *Imaeda T & Ogura M* Formation of intracytoplasmatic membrane system of mycobacteria related to cell division J Bact *85* 150 163 1963

7 *Klingmüller G & Orfanos C* Der electronenmikroskopische Aufbau des *Mycobacterium leprae* Arch klin exp Derm *224* 373–384 1966

8 *Koike M & Takeya K* Fine structure of intracytoplasmatic organelles of mycobacteria J biophys biochem Cytol *9* 597–608 1961

9 *Kappler W* Über die Verbreitung einer sauren Phosphatase innerhalb der Gattung Mycobacterium Beitr Klin Tuberk *129* 210–213 1964

10 *Kappler W* Zur Differenzierung von Mycobacterien mit dem Phosphatase Test Beitr klin Tuberk *130* 223 226 1965

11 *Kolbel H* Bemerkungen zum Aufbau der Zellwand von Mycobakterien Beitr Klin Tuberk *130* 203–209 1965

12 *Petitpre A Roos P & Tacquet A* Aspect ultrastructural de *Mycobacterium* phlei J Microscopie *6* 229–232 1967

13 *Reynolds E S* The use of lead citrate at a high pH as electron opaque stain in the electron microscopy J Cell Biol *17* 208 212 1963

14 *Ryter A & Kellenberger E* Etude au microscope électronique de plasmas contenant de l acide désoxyribonucleique I Les nucloides des bactéries en croissance active Z Naturforsch *13b* 597–605 1958

15 *Ryter A & Kellenberger E* L inclusion au polyester pour l ultra microtomie J Ultrastruct Res 2 200–214 1958

16 *Spicer S S Wetzel B K & Heppel L A* Fine structural cytochemistry of several phosphatases in *E coli* Fed Proc *25* 539 1966

17 *Takeya K Hisatsune K & Inoue Y* Mycobacterial cell walls II Chemical composition of the basal layer J Bact *85* 24–30 1963

18 *Urabe K Suto H Tasaka H & Matsubayashi A* Acid phosphatase activity of mycobacteria Amer Rev resp Dis *91* 279 282 1965

Acta path microbiol scandinav 74 61 68 1968

The National Bacteriological Laboratory Department of Bacteriology
Stockholm Sweden

PHAGE TYPING
OF *PSEUDOMONAS AERUGINOSA*

By

Lennart Sjöberg and Alf A Lindberg

Received 15 ii 68

Pseudomonas aeruginosa has been increasingly recognized as the aethiological agent of a variety of human infections in hospitals Paediatric patients and debilitated adults are particularly susceptible to infections with this organism The pathogenicity of *P aeruginosa* in premature infants is well known and reports on the spread of the bacteria with contaminated resuscitation equipment have been published (*Bassett Thompson & Page* 1965 *Fierer Taylor & Gezon* 1967) Primary infections of *P aeruginosa* in adults are less common but often serious Severe postoperative infections of the eye with panopthalmitis were caused by saline contaminated with this organism (*Lancet* 1966 *Ayliffe Barry Lowbury Roper Hall & Walker* 1966) Patients treated with a steroid ointment containing neomycin and amphomycin contaminated with *P aeruginosa* developed severe eye infections (*Kallings Ringertz Silverstolpe & Ernerfeldt* 1966 *British Medical Journal* 1967) Respiratory apparatus and rinse solution for tracheal catheters have been reported as sources of cross infections (*Phillips & Spencer* 1965 *Sutter Hurst Grossman & Calonje* 1966) Patients with severe burns often succumb in septicaemias caused by for example *P aeruginosa* (*Markley Gurmendi Chavez & Bazan* 1957 *Kefalides Arana Bazan Velarde & Rosenthal* 1964 *Muller* 1967)

Different methods have been used for the epidemiological tracing of *P aeruginosa* strains *Habs* (1957) described a serological method using agglutination reactions for the subdivision of this organism *Wahba* (1963) classified the strains with the aid of pyocine typing Bacteriophage typing has also proved to be useful (*Gould & McLeod* 1960 *Postic & Finland* 1961 *Graber Latta Vogel & Brame* 1962 *Lindberg Latta Moncrief & Brame* 1962 and 1963 *Sutter Hurst & Fennel* 1965

We are grateful to Colonel R B Lindberg for the supply of the *P aeruginosa* bacterial phages and their corresponding propogating strains Our thanks are also due to Professor L O *Kallings* for continuous advice concerning various aspects of this work

The skilled technical assistance of Miss *Lena Zetterman* and Mrs *Lena Canyakmaz* is gratefully acknowledged

Shooter Walker, Williams, Horgan Parker, Asheshov & Bullimore 1966 *Sutter & Hurst* 1966) The present paper reports the results obtained using a set of 18 phages in typing of 667 *P aeruginosa* strains in Sweden during an 18 month period

MATERIAL AND METHODS

Bacterial Strains

790 cultures were collected during 1966 and a six month period in 1967 from various hospitals in Sweden The cultures were identified as *P aeruginosa* according to *Jessen* (1965) Colonies with a characteristic morphology were picked and the bacteria were identified as *P aeruginosa* if they a) produced pyocyanin or b) produced a fluorescent pigment and the growth pattern corresponded to that of *P aeruginosa* nitrate and nitrite should be reduced gelatina should be liquefied growth should not occur at 5 C but at 42 C, acid should be produced from the breakdown of mannitol but not of sucrose adonitol dulcitol sorbitol or inositol

Origin of Phages and Propagating Strains

18 phages and their propagating strain (PS) were obtained from Colonel *Robert B Lindberg* US Army Surgical Research Unit Ft Sam Houston Texas

Propagating Strains

The 18 propagating strains of *P aeruginosa* were grown in Trypticase Soy Broth (TSB) Subcultures were stored on Trypticase Soy Agar (TSA) plates at room temperature Each propagating strain was lyophilized

Propagation of Phages

15 of the phages were propagated at 30 C with the soft agar layer technique (*Adams* 1959) using 1 5 per cent TSA in the bottom and 0 75 per cent TSA in the top layer For three of the phages 68 F 7 and M 6 propagation in broth at 37 C yielded higher titres The crude lysate was centrifuged at 2 500 g for 20 minutes The supernatant was removed and the bacteria were killed by addition of 1 0 ml of chloroform per 50 ml of supernatant The phage titres ranged between 10^7–10^{11} plaque forming units/ml The phage stocks were stored at +4 C The titres were quite stable over a range of 12 months after addition of gelatin (50 μg/ml) to the phages

Determination of Routine Test Dilution (RTD)

The RTD of each phage suspension was determined by titration against the propagating strain The phages were applied with a phage typing applicator (Biddulph & Co 26 Fenwich St Manchester 15 Great Britain) in tenfold dilution steps in TSB to a seeded lawn of a 5 hour TSB culture at 37 C The agar plates were incubated at 30 C overnight The highest dilution causing almost confluent lysis was chosen as the RTD This varied between a 10^{-3} to a 10^{-6} dilution of the phage stock

Phage Typing Technique

Petri dishes containing 1 5 per cent TSA were sown with a 5 hour TSB-culture of the strain grown at 37 C A drop of each phagesuspension at RTD was applied with the phage typing applicator and the plates were incubated at 30 C overnight The reactions were recorded as +++ (confluent or almost confluent lysis) ++ (more than 30 isolated plaques) + (10 30 isolated plaques ± (less than 10 isolated plaques) Each *P aeruginosa* strain was characterized by the +++ and ++ reactions The isolated plaques produced by some of the phages (21 68 119X M6) on their propagating strains were of a small size but other phages (2 7 31 352 M4) yielded large plaques that gave almost confluent lysis with 30 plaques

TABLE I

Lytic Spectra of Pseudomonas aeruginosa Phages

Strain	Phages 2	7	16	21	24	31	44	1214	68	73	109	352	119X	17	F8	F10	M4	M6
2		4	5	5		3	5	5	5	5	5	1	5	3	5	3	5	
7	4	–										5	4				5	
16			–															
21	2			–						3	5		5				2	
24							5	5			5						4	
31				4		–				4								
44				5			–	5					5	5	3			2
1214		4	2	5			3	–	5	3	3	3			3	2	3	
68			1					5	–		5						2	
73			2			2			4	–			5	5			2	5
109					3			4			–				3			
352	5	5	3			5			5		5	–			4			
119X	5	5		5	5		2		5		4	5	–				5	2
17			2	5			1		5		3			–			2	
F8	4	2	5		5	2	5	5	5		5		5		–		4	4
F10			5													–	–	
M4	3	4	5	5	2	2	5	5	5	3	5	3	5	3	4		–	
M6			2	5	2	2	2	5	5		2		4	2		2	2	–

= a ++ reaction in the same dilution as on the homologous strain

= a ++ reaction in the dilution 10–10² times more concentrated than that giving ++ reaction on the homologous strain

= ibid but in the dilution 10³–10⁴ times more concentrated

= ibid but in the dilution 10⁵–10⁶ times more concentrated

= very weak lysis

= inhibition of growth

EXPERIMENTAL

Determination of the Lytic Spectrum

The lytic spectrum was determined according to the method used for Staphylococci (*Blair & Williams* 1961) using the phage typing applicator (Table 1). The phages were diluted in tenfold dilution steps in TSB and then tested against all the 18 propagating strains. The dilution giving minimal + + reaction (more than 10 isolated plaques) on the various propagating strains was compared with the dilution giving the same reaction on the homologous strain (Table 1).

Most of the phages lysed the *P. aeruginosa* strains 2 and M4, the reactions were recorded from 2 to 5. Seven of the phages showed reactions coded as 4 or 5 with 5 or more strains except for the homologous reaction. Phage F 10 lysed three strains but only with concentrated dilutions. All phages tested showed some reactions which were recorded as inhibition. This reaction was characterized by a thinning of the bacterial growth in the area were the phage had been applied. In these cases no isolated plaques could be seen with the various concentrations of the phage.

Phage Typing of 790 P. aeruginosa Cultures

790 cultures of *P. aeruginosa* isolated from patients in various hospitals were typed using the phage set at the RTD. The cultures were isolated from patients suffering from different infectious disorders to give a representative distribution of *P. aeruginosa* strains. Cultures isolated from the same patient and showing the same phage pattern were regarded as one strain. In total 667 strains were investigated (Table 2). 611 strains (91.6 per cent) were lysed by one or more phages whereas 56 strains (8.4 per cent) were not typable (NT).

TABLE 2

Phage Typing of 667 Pseudomonas aeruginosa *Strains*

		Number of strains	Percentage typable	Percentage not typable
National Bacteriological Laboratory	miscellaneous infections	262	93.1	6.9
Danderyds sjukhus	miscellaneous infections	197	93.4	6.6
Akademiska sjukhuset *Uppsala*	miscellaneous infections	1[illegible]	91.0	9.0
Karolinska sjukhuset Stockholm	chronic urinary tract infections	43	88.4	11.6
Malmö Allmänna sjukhus	appendicitis	43	79.1	20.9
Total		667	91.6	8.4

The organisms obtained at the National Bacteriological Laboratory, Stockholm, were isolated from urine, pus, sputum and faecal cultivations from patients in different hospitals and from outclinic patients. A total of 243 strains (93.1 per cent) were lysed whereas 19 (6.9 per cent) were NT. With the aid of the phages the organisms were separated into more than 160 different lysis patterns.

At Danderyd's hospital, a central county hospital, 197 strains were isolated in different wards. Of these 184 (93.4 per cent) were lysed. The organisms were separated into 129 lysis patterns.

A total of 111 strains (91.0 per cent) from the Akademiska sjukhuset at Uppsala, a medical school hospital, were separated into 64 different lysis patterns. The number of NT strains was 11 (9.0 per cent). Eight NT cultures were isolated from one patient during a 2 month period.

From a renal ward at the Karolinska sjukhuset in Stockholm, a medical school hospital, 43 strains were collected. The microorganisms were isolated from 23 patients, all suffering from chronic urinary tract infections. Among the strains, 38 (88.4 per cent) were lysed whereas 5 (11.6 per cent) were NT. The microorganisms were separated into 36 different phage patterns.

The material from Allmänna sjukhuset in Malmö, a medical school hospital, consisted of 43 *P. aeruginosa* isolations from the appendix of patients with appendicitis. Out of these strains, 34 were lysed and separated into 30 different phage patterns.

The recording of the results was easy for most cultures as the phages showed strong lytic reactions. As a rule the phage pattern of each strain was determined by at least 3 and rarely more than 10 lytic reactions. As regards some strains, difficulties were met when the reactions of phages 31, 73 and 119X were to be recorded. The bacterial lawn where the phage drop had been placed and a surrounding zone were more translucent than the rest of the bacterial lawn. On dilution of the phages no isolated plaques were found and therefore these reactions were not regarded as positive.

Retyping of 424 P. aeruginosa Cultures

After storage for more than 4 months, 424 of the cultures preserved as agar stab cultures were retyped. The difference between the phage patterns obtained on the two occasions was recorded. A difference between a + + + and a + + reaction in the two typings was not considered as significant. However, if the reaction had changed from + + + to + or to no lytic reaction at all, this difference was recorded as well as the reverse phenomenon. Thus a difference in reaction of more than one + was recorded as significant.

No significant difference in the lytic patterns of 64 per cent of the cultures was observed. One phage reaction was found to be different in

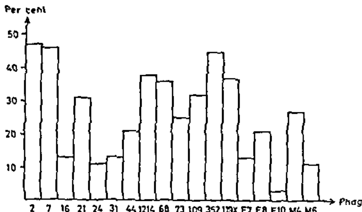


Fig 1

The frequency of lytic reactions for each phage

27 per cent of the cultures Two differences were found in 6 per cent and three in 3 per cent of the cultures None of the cultures differed in reaction with more than three phages on the two occasions of typing

The Frequency of Lytic Reactions for Each Phage

The frequency of lytic reactions for each phage on the 790 cultures is presented in Figure 1 Only + + + and + + reactions were recorded Three phages 2 7 and 352 lysed more than 40 per cent each of the cultures 5 phages lysed 30 per cent of the cultures Only one of the phages F10 lysed less than 10 per cent of the cultures

DISCUSSION

The increasing frequency of *Pseudomonos aeruginosa* infections in hospitals calls for effective methods for the epidemiological tracing of this organism Using the phage set of 18 different phages of *Lindberg* (1961) a total of 611 out of 667 strains (91 6 per cent) could be separated into different lysis patterns in the present investigation The microorganisms examined consisted of isolations from different sources and hospitals over a period of more than 18 months (Table 2)

The frequency of nontypable strains in the different materials varied between 6 6 *per cent* and 20 9 per cent Throughout the investigation *the phages* were used only in the routine test dilution the phage stocks were diluted from 1 1000 to 1 100 000 With the use of more concentrated phage dilutions for example 1000 × RTD the frequency of *typable strains* was increased The phages however frequently showed inhibition reactions when used in higher concentrations When reactions at 1000 × RTD were recorded it was often difficult to distinguish between a *true lysis* reaction and an inhibition reaction In such cases

it was necessary to test the phage in the dilution steps between 1000 × RTD and RTD As more than 90 per cent of the strains in this investigation were lysed at RTD the use of the phages at 1000 × RTD was not considered necessary

When the lysis reactions were recorded the limit for a + + reaction which was the lowest reaction reported in the lysis pattern was put at more than 30 plaques as about 30 plaques formed by the phages 2 7 31 352 and M4 produced almost confluent lysis in the test spots Since such a small number of plaques were used to establish the typing it was necessary to standardize the typing method to a high degree to obtain reproducible results

The stability of the lysis patterns was good when the strains had been stored for more than 4 months The majority of the strains 91 per cent did not differ in more than one strong lytic reaction The lytic spectrum of the phages also has been of the same degree of stability when tested on different occasion with newly propagated phage stocks

The host range of the phages used in this test was broad enough to separate more than 90 per cent of the strains To obtain an international basic set however it will be necessary to compare the lytic spectrum of these phages with that of the phages used by other investigators Several investigators using other phage sets have reported that the frequency of typable strains of *P aeruginosa* was high The number of lysed strains have varied between 68 per cent and 92 5 per cent (*Gould et al* 1960 *Postic et al* 1961 *Graber et al* 1962 *Feary Fischer & Fischer* 1963 *Lindberg et al* 1964 *Meitert* 1965 and *Sutter et al* 1965) It ought to be possible to obtain a standardized international phage set to be used for the phage typing of *P aeruginosa* as has been done for the phagetyping of *Staphylococcus aureus* comprising phages both with a broad and a more specific host range

SUMMARY

Cultures identified as *Pseudomonas aeruginosa* were typed using a phage set of 18 *P aeruginosa* bacteriophages The lytic spectra of the phages were determined With this set of phages 611 out of 667 (91 1 per cent) strains of *P aeruginosa* were lysed with a variation from 79 per cent to 93 per cent typable strains in five different materials As a rule the strains were lysed by from 3 to 10 different phages and subdivided into different distinct lysis patterns The stability of the lysis patterns on repeated typings was found to be good The phage set gave a suitable subdivision of *P aeruginosa* strains for epidemiological typing

REFERENCES

Adams M H The Bacteriophages Interscience Publishers New York 1959

Ayliffe G A J Barry D R Lowbury E J L Roper Hall M J O & Walker W M Postoperative Infection with *Pseudomonas aeruginosa* in an Eye Hospital Lancet 1 1113 1117 1966

Bassett D C J Thompson S A S & Page B Neonatal Infections with *Pseudomonas aeruginosa* Associated with Contaminated Resuscitation Equipment Lancet 1 781–784 1965

Blair J E & Williams R E O Phage Typing of Staphylococci Bull Wld Hlth Org 24 771–784 1961

British Medical Journal Contamination of Pharmaceutical Products (leader) 2 125–126 1967

Feary T W Fischer E & Fischer T N Lysogeny and Phage Resistance in Pseudomonas aeruginosa Proc Soc Exp Biol and Med 113 426–430 1963

Fierer J Taylor P M & Gezon H M Pseudomonas aeruginosa Epidemic Traced to Delivery Room Resuscitators New Engl J Med 276 991–996 1967

Gould J C & McLeod J W A Study of the use of Agglutinating Sera and Phage Lysis in the Classification of Strains of *Pseudomonas aeruginosa* J Path Bact 79 295–311 1960

Graber C D Latta R Vogel E H & Brame R Bacteriophage Grouping of *Pseudomonas aeruginosa* with Special Emphasis on Lysotypes Occurring in Infected Burns Am J Clin Path 37 54–62 1962

Habs I Untersuchungen uber die O Antigene von *Pseudomonas aeruginosa* Z Hyg Infekt Kr 144 218–228 1957

Jessen O Pseudomonas aeruginosa and other green fluorescent Pseudomonas—A taxonomic study Munksgaard Copenhagen 1965

Kallings L O Ringertz O Silverstolpe L & Ernerfeldt F Microbiological Contamination of Medical Preparations Acta Pharm Suecica 3 219–228 1966

Kefalides N A Arana J A Bazan A Velarde N & Rosenthal S Evaluation of Antibiotic Prophylaxis and Gamma Globulin Plasma Albumin and Saline Solution Therapy in Severe Burns Bacteriologic and Immunologic Studies Ann Surg 159 496–506 1964

The Lancet Pseudomonas aeruginosa (leader) 1 1139 1140 1966

Lindberg R B Latta R L Moncrief J A & Brame R E Serodiagnosis and Bacteriophage Typing of Pseudomonas Ann Progress Report 1961–1962 1 11

Lindberg R B Latta R L Brame R E & Moncrief J A Bacteriophage Typing of *Pseudomonas aeruginosa* Epidemiologic Observations based on type distribution Ann Progress Report 1962–1963 1 19

Lindberg R B Latta R L Brame R E & Moncrief J A Definitive Bacteriophage Typing System for *Pseudomonas aeruginosa* Bact Proc 15 81 1964

Markley K Gurmendi G Chavez P M & Bazan A Fatal Pseudomonas Septicemias in Burned Patients Ann Surg 145 175–181 1957

Meitert E Schéma de Lysotypie pour *Pseudomonas aeruginosa* Arch Roum Path Exp Microbiol 24 439–458 1965

Muller E F Gentamicin in the Treatment of Pseudomonas Infections Complicating Burns Gentamicin First International Symposium. Paris January 1967 Schwabe & Co Basel p 148–153

Phillips I & Spencer G Pseudomonas aeruginosa Cross Infection Due to contaminated Respiratory Apparatus Lancet 2 1325–1327 1965

Postic B & Finland M Observations on Bacteriophage Typing of *Pseudomonas aeruginosa* J Clin Invest. 40 2064–2075 1961

Shooter R A Walker K A Williams V R Horgan G M Parker M T Asheshov E H & Bullimore J F Faecal Carriage of *Pseudomonas aeruginosa* in Hospital patients Possible spread from patient to patient Lancet 2 1331–1334 1966

Sutter V L Hurst V & Fennell J A Standardized System for Phage Typing *Pseudomonas aeruginosa* Health Lab Sci 2 7–16 1965

Sutter V L & Hurst V Sources of *Pseudomonas aeruginosa* Infection in Burns Study of Wound and Rectal Cultures with Phage Typing Ann Surg 163 597–602 1966

Sutter V L Hurst V Crossman M & Calonje B Source and Significance of *Pseudomonas aeruginosa* in Sputum Patients Requiring Tracheal Suction J A M A 197 854–858 1966

Wahba A H The Production and Inactivation of Pyocines J Hyg (London) 61 431–441 1963

Acta path. microbiol. scandinav. 74: 69–76, 1968

The Streptococcus Department, Statens Seruminstitut, Copenhagen
(Chief: Kn. Skadhauge, M.D.) and the Medical Department, Hillerød County Hospital
(Chiefs: P. Grønbæk, M.D. and M. Iversen, M.D.), Denmark.

GROUP R STREPTOCOCCI PATHOGENIC FOR MAN

Two Cases of Meningitis and one Fatal Case of Sepsis

By

Beate Perch, P. Kristjansen and Kn. Skadhauge

Received 21.ii.68

In 1963 *de Moor* (2) reported studies on streptococci isolated from septicaemic infections in pigs. These streptococci grew on horse blood agar with colonies surrounded by a partial zone of haemolysis resembling the haemolysis characteristic for Group B streptococci. A few strains showed no or very weak haemolysis on horse blood agar. On sheep blood agar all strains were found to be non-haemolytic. *De Moor* has not reported any investigations dealing with the ability to form a soluble haemolysin. Biochemically these streptococci were especially characteristic by their ability to split inulin and their resistance to 40 per cent bile. Serologically the strains examined did not belong to the known Lancefield Groups A to Q. However, by precipitation with formamide extracts antigens were demonstrated which permitted differentiation into three serological groups. *De Moor* designated these serological groups R, S and T. A few strains were precipitated by antisera of both Group R and Group S (RS streptococci). The antisera employed also precipitated extracts of Group D. These overlapping reactions were, however, removed by absorption with *Streptococcus faecalis* bacteria.

Human infections with streptococci belonging to *de Moor's* Groups R, S and T are not reported in the literature. *De Moor* suggests that these streptococci apparently are confined to pigs. He did not succeed in finding streptococci of Groups R, S and T in a material of about 2,000 strains isolated from monkeys, cattle, sheep, horses, dogs, cats, rabbits, mice and guinea pigs, nor have they been found in man.

During the period 1960 to 1966 we have diagnosed Group R streptococci in three cases of severe human infection, *viz.* two cases of meningitis in which streptococci were isolated in pure culture from the spinal fluid (in one of the cases also from blood), and one fatal case of sepsis in which streptococci were isolated in pure culture from the spinal

Thanks are due to Dr. *Erna Lund* who kindly put her Pneumococcus sera at our disposal.

fluid and from blood An account is given here of the case histories and the bacteriological analysis of the Group R streptococci isolated

CASE HISTORIES

Case B

J no B 1893/62-63 61 year old man works manager at a sawmill Previously generally healthy Six days before admission to hospital the patient sustained a slight burn on the right side of the face from a spurt of flame from the central heating oven Slight *redness developed but disappeared rapidly Two days before* admission again redness and oedema of the right cheek with slight blister formation

Four days before admission severe attacks of shivering with temperature elevated to 39 5 C During the subsequent days septic temperature variations and increasing severe diffuse headache One day before admission the patient developed stiffness of the neck and the sensorium became affected Penicillin was prescribed by the patient s own doctor and administered for two days

At admission the patient was exhausted febrile in pain and with pronounced stiffness of the neck

Lumbal puncture was *performed at once The spinal fluid was turbid and yel*lowish Microscopy revealed 19 776/3 leucocytes mainly granulocytes with polymorphous nuclei protein content 491 mg per cent No bacteria were demonstrated by direct microscopy

Moderate oedema redness and warmth of the right half of the face were also observed at admission and one or two small bullae containing clear fluid were situated in the middle of the cheek The processes spread during the first days but disappeared after five to six days Culture from the skin was not made

Treatment with penicillin streptomycin and sulphadiazine was instituted primarily

On the second day after admission it was notified from the Statens Seruminstitut that culture from the spinal fluid had shown growth of streptococci These were later identified as haemolytic streptococci Group R The bacteria were sensitive to all the antibiotics examined with the exception of the sulpha preparations Treatment with these was consequently discontinued During the first six days in hospital the patient was deeply unconscious and had several attacks of tonic and clonic convulsions lasting for a minute or two

The temperature gradually became normal and the patient slowly regained consciousness During the first period he was fumbling confused and restless He complained of deafness and an audiogram showed severe impairment of hearing of the perception type It was considered that this impairment was due to the meningitis and not to the streptomycin treatment since only a total of 10 g of streptomycin had been administered After just over one month and a half in *hospital the patient was discharged No definite psychic reduction could be de*monstrated and the hearing was somewhat better

Case L

J no F 1574/66-67 38 year old slaughterhouse worker Apart from tendency to migraine previously mainly healthy

A few days before admission to hospital the patient sustained a slight injury to the right wrist during his work having scratched himself on the rib of a slaughtered *pig* One or two days later he developed pain in the right upper extremity redness and tenderness around the site of injury and a little purulent secretion from the wound

Four days before admission the patient suddenly developed severe attacks of shivering and rise in temperature to about 40 C During the subsequent days there were severe temperature *variations together* with nausea vomiting and severe diffuse headache

Treatment with penicillin was prescribed by the patient s own doctor

At admission the patient was highly febrile confused fumbling and motorically extremely restless There was pronounced stiffness of the neck and positive Kernig s sign

Lumbal puncture gave turbid milky spinal fluid and microscopy showed 4 964/3

leucocytes 90 per cent of which were granulocytes protein content 4?0 mg per cent Direct microscopy revealed numerous bacteria thought to be pneumococci

The wound on the wrist was dirty with a little purulent secretion and there was redness of the area around it Culture from the wound was not performed The lesion healed in the course of a few days

Treatment was instituted primarily with penicillin streptomycin and sulphadiazine On the third day notification was received from the Statens Seruminstitut that culture from the spinal fluid and blood gave growth of non haemolytic streptococci belonging to Group B These were sensitive to all the antibiotics examined with the exception of the sulpha preparations treatment with which was then discontinued

During the first days of the stay in hospital the patient was extremely ill with hyperthermia This condition was treated for a time with cold blankets and truxal with good effect Furthermore the treatment was supplemented by act ... and for a time by chloramphenic l and erythromycin

X ray of the thorax at admission showed no abnormality Four days later there were pronounced bilateral pneumonic processes These decreased considerably in the course of a few days

Ophthalmoscopy showed slight blurring of the papillae without venestasis

The temperature became normal after 7 days and the patient began to recover slowly During the first days he was somewhat confused and had hallucinations but these disappeared quickly He complained of impaired hearing and dizziness The latter symptom disappeared spontaneously but the hearing remained impaired The patient stated that before the present illness he did not hear well (noisy work)

After one month in hospital the patient was discharged in a good state of health

Case N[1]

J no 16037/60 44 year old man admitted with symptoms of septicaemia or toxic shock Death occurred a few hours after admission Information is available that the patient had had an infected wound and that he had been ill for some time before admission *Post mortem* examination revealed pronounced cadaverosis such as that found in patients with septicaemia. In addition there were small yellow infiltrates in the liver (probably small abscesses) Culture from spinal fluid and blood gave growth of bacteria and the probable diagnosis is therefore fulminant sepsis emanating from the infected wound

Besides the pathogenesis there are several similarities in the case histories of the two patients B and I The courses of the disease were almost identical Furthermore it is possible that the origin of the infection in Cases B and I as well as in Case N was the minor skin lesion sustained a few days before the onset of the disease

MATERIAL

In all seven strains were studied one strain from Case B (spinal fluid) three strains from Case L (one from spinal fluid two from blood taken at different times on the same day) and three strains from Case N (one from spinal fluid two from blood taken at different times on the same day) As reference strain Strain 735 isolated from pig (?) was employed

METHODS

The following *biochemical* and *cultural* procedures were used

1) Growth on 1 8 per cent beef broth agar containing 5 per cent defibrinated horse blood

[1] The writers are grateful to Dr *Michael Schwartz* Chief Physician Medical Department County Hospital Glostrup for permission to report this case

2) Growth on 1 4 per cent beef broth agar containing 8 per cent defibrinated horse blood and 40 per cent ox bile
3) Growth on 1 8 per cent ox agar containing 10 per cent horse blood (mixed at 80 C for about 5 minutes) and 0 04 per cent potassium tellurite
4) Growth in broth containing 6 5 per cent sodium chloride
5) Growth in broth at 45 and 10 C.
6) Final pH after growth for 8 days in 1 per cent glucose broth
7) Production of ammonia from L-arginine hydrochloric acid demonstrated by Nessler's reagent
8) Hydrolysis of sodium hippurate shown by the addition of 50 per cent sulphuric acid to two parts of the supernatant broth culture
9) Mucus formation on growth on 1 8 per cent beef broth agar containing 5 per cent sucrose
10) Production of acid during growth for 8 days in broth containing the drugs recorded in Table 1 using Difco broth base and phenol red as indicator
11) Hydrolysis of esculin and glycogen using Difco broth base and of starch using oxoid nutrient agar No 2 containing 0 2 per cent soluble starch
12) Camp reaction according to *Christie et al* (1)
13) Test for soluble haemolysin using rabbit erythrocytes (5 per cent)
14) Sensitivity to sulphathiazole and the antibiotics given in Table 1 measured according to *Thomsen* (7)

Serological Methods

The streptococci were harvested from growth in trypsin digested broth for 18 hours at 36 C

Antisera were prepared in rabbits by repeated intravenous injections of a saline suspension of heat killed (60 C for 30 minutes) and three times washed bacteria. They were stored at 4 C in a dense suspension (1 100 of the original volume) and preserved by adding 0 5 per cent formalin

Antigen for absorption was prepared in the same way as the antigen used for immunization and centrifuged at 12 000 r p m About 1 3 to 1/2 volume of packed cells was added to undiluted serum and allowed to bind for one to two hours at 36 C in some cases followed by further binding at 4 C overnight

Agglutination tests were carried out in tubes with culture (a) killed by 0 2 per cent formalin and (b) killed by 100 C for two hours and read after 20 hours in water bath at 50 C

Neufeld reaction was carried out on slides by mixing a loopful of a 6 and an 18 hour culture with a loopful of serum Reading was made immediately after the mixing

Acid extracts were made according to *Lancefield* (5) (using 0 1 n hydrochloric acid) *formamide extracts* according to *Fuller* (4) and *saline extracts* according to *Elliott* (3)

Precipitin tests were carried out in microtubes with a diameter of 1 5–2 0 mm

RESULTS

Biochemical and Cultural Procedures

Morphological examination of Gram stained smears from fluid media showed cocci arranged in pairs and short chains Uniform turbidity was obtained in fluid media On horse blood agar the colonies had a glistening surface and a narrow zone of β haemolysis On prolonged incubation they resembled Group B haemolytic streptococci as already observed by *de Moor* (2) except for the α haemolytic strains isolated from one of the patients (Case I) All the strains that formed β haemolysis on blood agar produced a soluble haemolysin

Table 1 shows the cultural and biochemical reactions of Group B streptococci isolated from three patients (B L N) and the reference

TABLE 1

Biochemical and Cultural Behaviour of Human Group B Streptococci Compared with the Animal Reference Group B Strain 73a

	De Moor B 73a	Case N strains N_1 N_2 N_4	Case L strains L_1 L_2 L_3	Case B strain
Acid formation in				
Glucose sucrose lactose maltose salicin trehalose raffinose inulin melibiose	+	+	+	+
Arabinose mannitol sorbitol glycerol melezitose	—	—	—	—
Hydrolysis of				
Esculin glycogen starch	+	+	+	+
Arginin	+	+	+	+
Sodium hippurate	—	—	—	—
Resistance to				
Ox bile	+	+	+	+
Tellurite	—	—	—	—
NaCl	—	—	—	—
45° C	—	—	—	—
Mucus formation	—	—	—	—
Horse blood agar	β	β	α	β
Soluble haemolysin	+	+	—	+
Camp reaction	(+)	(+)	(+)	(+)
Sensitivity to sulphathiazole	—	—	—	—
Sensitivity to				
Penicillin	+++	+++	+++	+++
Streptomycin	++	+	++	++
Tetracycline	+++	+++	+++	+++
Chloramphenicol	+++	+++	+++	+++
Erythromycin	+++	+++	+++	+++
Bacitracin	+	0	+	0

Varies from — to + in different tests

Group B Strain 73a isolated from a pig. It will be seen that the findings were the same both with the animal strain and the human strains.

Serological Examinations

One strain from each patient was included in the examinations. Antisera were prepared from strains isolated from two of the three patients, viz. representatives of an α haemolytic and a β haemolytic strain (L and B) and the animal reference Strain 73a.

It will be seen from the results recorded in Table 2 that the human α and β haemolytic streptococci which reacted in antisera against the animal reference strain of Group B are apparently serologically identical and identical with the animal Group B Strain 73a.

The reactions of all strains in unabsorbed and absorbed sera were equally strong and negative respectively. In cross absorption experi

TABLE 2

Serological Examinations with Unabsorbed and Absorbed Immune Sera of the Present Group R Streptococci

Capsular titre	Antisera against R 735	Antisera against B	Antisera against I	Undiluted antisera of R 735 absorbed with B	B absorbed with R 735	R 735 absorbed with I	L₂ absorbed with R 735	I absorbed with B	R 735 absorbed with N	Rabbit pre immune serum
R 735	20	20	40	—	—	—	—		—	< 2
B	20	20	40	—	—			—		< 2
L₂	20	20	40			—	—	—		< 2
N	20	20	40						—	< 2
Tube agglutination titre using formalin killed or heat (127 C) killed antigens										
R 735	*80*	*160*	*160*	—	—	—	—		—	<10
B	80	160	320	—	—			—		<10
L	80	160	160			—	—	—		<10
N	80	160	160						—	<10
Precipitation with acid extracts										
R 735	+++	+++	+++	—	—	—	—		—	—
B	+++	+++	+++	—	—			—		—
L	+++	+++	+++			—	—	—		—
N	+++	+++	+++							—
Precipitation with formamide extracts										
R 735	(+++)	(+++)	(+++)	—	—	—	—		—	—
B	(+++)	(+++)	(+++)	—	—			—		—
I	(+++)	(+++)	(+++)			—	—	—		—
N	(+++)	(+++)	(+++)						—	—
Precipitation with saline extract using living (2 hours 36 C) or heated (2 hours 127 C) bacteria										
R 735	d	d	d	—	—	—	—		—	—
B	d	d	d	—	—			—		—
I	d	d	d			—	—	—		—
N	d	d	d						—	—

+++ = prompt precipitation
(+++) = prompt but weak precipitation
d = firm disk shaped precipitate
— = no reaction

ments with undiluted sera all precipitins and agglutinins were removed from sera prepared against the animal strain and the human strains

Non concentrated filtrates of an 18 hour glucose broth culture gave a prompt precipitation reaction with homologous and heterologous anti sera More concentrated preparations obtained by saline extraction of living organisms according *to the method indicated by Elliott* (3) for capsulated PM streptococci and by extraction at 127 C gave a firm disk shaped precipitate

The swelling reaction in the presence of type specific immune sera was comparable with that observed with *Streptococcus pneumoniae* The reaction was equally strong when 6 and 18 hour cultures were used

In accordance with the findings obtained by *de Moor* (2) acid ex

tracts using 0 2 n hydrochloric acid could not be precipitated The form amide extracts gave weak reactions independent of the temperature used (100 120 140 and 160 C) The reason may be that the carbo hydrate antigen is partly precipitated by the precipitation with acid ethanol This will be examined in details elsewhere

DISCUSSION

Group R streptococci are reported here for the first time as the cause of meningitis and sepsis in man

The isolated strains were culturally biochemically and serologically identical with the Group R streptococci isolated from porcine infections in The Netherlands by *de Moor* (2) The animal strain and the human strains from two of the three cases were β haemolytic capsulated strep tococci while the strains from the third case were α haemolytic cap sulated streptococci All strains gave a positive Camp reaction How ever the demonstration of soluble haemolysin and the Camp reaction failed in some tests Neither the animal nor the human strains gave signs of being pathogenic or toxic for white mice as indicated from several passages in mice No relationship between the capsule of the present strains and *Streptococcus pneumoniae* was found when the capsular antigen of the present Group R streptococci in sera of *Strep tococcus pneumoniae* Types 1 to 48 were tested by the Neufeld reaction

Recently *Elliot* (3) reported capsulated β haemolytic streptococci containing the Group D antigen as the cause of neonatal infection in piglets in England These streptococci appear to be identical with those isolated from piglet infections by *de Moor* who designated his strains Group S (cited *Elliot*) *De Moor* (2) had already drawn attention to overlapping reactions between Groups R S and T streptococci and *Streptococcus faecalis*

In the present examinations no evidence was found of the existence of Group D antigen in Group R streptococci either by precipitin tests with acid extracts in antisera prepared by prolonged immunization with the present strains or in Group D sera from The Wellcome Labor atories Beckenham England

In single antisera of Group B Strain O 90 and Group R streptococci common antibodies for B O 90 and Group R could be demonstrated Cross absorptions indicated however that the group antigens in Groups B and R were not identical

The finding of streptococci of Lancefield Group R in cases of human infection in Denmark seems to be particularly remarkable since as far as is known such streptococci have not been demonstrated in animals in this country

TABLE 2

Serological Examinations with Unabsorbed and Absorbed Immune of the Iresent Croup R Streptococci

Capsular titre	Antisera against			Undiluted antisera of R 735 absorbed with	B absorbed with	R 735 absorbed with	I absorbed with	I absorbed with	R 735 absorbed with	ii s
	R 735	B	I	B	R 735	I	R 735	B	N	
R 735	20	20	40	—	—	—	—		—	<
B	20	20	40	—	—			—		<
I_2	20	20	40			—	—	—		<
N_2	20	20	40						—	<
Tube agglutination titre using formalin killed or heat (127 C) killed antigens										
R 735	80	160	160	—	—	—	—		—	<10
B	80	160	320	—	—			—		<10
L	80	160	160			—	—	—		<10
N	80	160	160						—	<10
Precipitation with acid extracts										
R 735	+++	+++	+++	—	—	—	—		—	—
B	+++	+++	+++	—	—			—		
I	+++	+++	+++			—	—	—		—
N	+++	+++	+++							—
Precipitation with formamide extracts										
R 735	(+++)	(+++)	(+++)	—	—	—	—		—	—
B	(+++)	(+++)	(+++)	—	—			—		—
I	(+++)	(+++)	(+++)			—	—	—		—
N	(+++)	(+++)	(+++)						—	—
Precipitation with saline extract using living (2 hours 36 C) or heated (2 hours 127 C) bacteria										
R 735	d	d	d	—	—	—	—		—	—
B	d	d	d	—	—			—		—
I	d	d	d			—	—	—		—
N	d	d	d						—	—

+++ = prompt precipitation
(+++) = prompt but weak precipitation
d = firm disk shaped precipitate
— = no reaction

ments with undiluted sera, all precipitins and agglutinins were removed from sera prepared against the animal strain and the human strains

Non concentrated filtrates of an 18 hour glucose broth culture gave a prompt precipitation reaction with homologous and heterologous antisera More concentrated preparations obtained by saline extraction of living organisms according to the method indicated by *Elliott* (3) for capsulated PM streptococci and by extraction at 127 C gave a firm disk shaped precipitate

The swelling reaction in the presence of type specific immune sera was comparable with that observed with *Streptococcus pneumoniae* The reaction was equally strong when 6 and 18 hour cultures were used

In accordance with the findings obtained by *de Moor* (2) acid ex

Acta path microbiol scandinav 74 77-84 1968

The Enterovirus Department Statens Seruminstitut Copenhagen Denmark

DEVELOPMENT AND PERSISTENCE OF NEUTRALIZING ANTIBODY IN HUMAN RUBELLA INFECTION

By

JØRGEN LEERHØY

Received 21 ii 68

Rubella occurred in epidemic proportions in Denmark during the spring of 1964 To obtain informations about the development and persistence of neutralizing antibody during infection with rubella virus a number of serial serum samples was collected The study reported here is based on a series of 9 rubella patients bled at intervals from onset of illness over periods covering from one to three years and a half

Upon collection all sera were routinely heat inactivated at 56° C for 30 minutes a procedure which later was shown to reduce rubella neutralization titres considerably (10 11 12) In a previous report (6) the antibody potentiating action of normal guinea pig serum (GPS) on the neutralization of rubella virus in heated human sera has been described In the present study the heated human sera have been examined with and without the addition of normal GSP

MATERIALS AND METHODS

Human sera The sera were obtained from 9 patients with a clinical diagnosis of rubella with rash The material comprises 7 female and 2 male patients aged from 18 to 39 years at onset of illness Specimens were collected at intervals over a period of 1 1/2 to 15 months additional blood sample were drawn from 6 patients three years and a half after the onset of illness An attempt was made to obtain blood specimens at regular weekly intervals for the first two months and thereafter at 6 12 and 15 months ending with a specimen drawn three years and a half after infection

The sera were heat inactivated at 56° C for 30 minutes upon collection and stored at minus [illegible]0° C until tested

Neutralization tests The neutralization tests were performed in SIRC cells in accordance with the technique previously described (6) All sera were tested with and without the addition of [illegible] per cent GPS When a series of individual sera from a patient has been titrated in more than one experiment the results have been linked

The author wishes to extend his sincere thanks to Dr *Herdis von Magnus* who initiated the collection of the sera employed in this study and later placed them at the authors disposal The patients were kindly referred to us by the Mothers Aid Centre and the Department of Contageous Diseases of the Blegdamshospital Copenhagen and by Dr *H Bennedsen* Svendborg

The skilful technical assistance of Mrs *D Lindvall* and Mrs *H Lindhard* and the assistance of Miss *G Madsen* R N in bleeding the patients is gratefully acknowledged

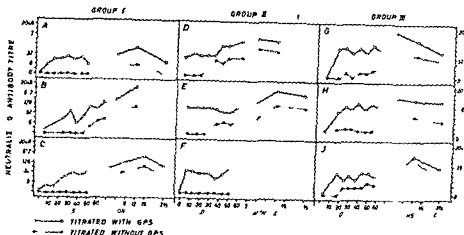


Fig 1

Individual antibody response curves for rubella patients A to J in heat inactivated sera titrated with and without guinea pig serum (GPS)

together by retitrations of previously tested sera of the same series as well as by the inclusion of a local reference serum Titres have been expressed as the reciprocal of the initial serum dilution

Throughout this paper titres less than 4 are recorded as 0

RESULTS

In the description of the results the patients examined have been divided into three groups according to similarities in the antibody patterns The results are illustrated in Fig 1 and summarized in Table 1 All sera had as mentioned above been heat inactivated and accordingly two sets of antibody titres are recorded for each patient one set representing the titrations performed without addition of GPS and the other set with 2 per cent GPS The sera thus titrated and the titrations in which they are tested are in the following referred to as GPS free and GPS supplemented sera or titrations respectively

Group I comprising patients A B and C shows antibody curves which are characterized by early appearance of antibody in the GPS supplemented sera with an even gradual rise in titre while the GPS free titrations at the earliest demonstrate neutralizing antibody 6 to 7 weeks after rash Maximal titres appear late 12 to 15 months or perhaps later after onset of illness

In the GPS supplemented sera taken from patients A and C at the day of rash no antibody is demonstrable During the first week of illness however all patients in the group develop antibody titres to a level of 4 to 8 A steady but rather slow increase to titres from 32 to 180 takes place in patients B and C during the first two months while for patient A the antibody curve after two weeks seems to level off at a titre around 16 All patients reach maximal titres of 90 to 1450 12 to 15 months after illness The patients from whom blood specimens are

TABLE 1

Rubella Neutralising Antibody Titres in Individual Sera A–J

Group	Patient	With 2 per cent Guinea Pig Serum: Antibody first demonstrated, day	titre	Maximal titre, day	titre	Titre 3½ year after illness	Without Guinea Pig Serum: Antibody first demonstrated, day	titre	Maximal titre, day	titre	Titre 3½ year after illness
I	A	7	8	376	90	11	187	6	376	8	0
	B	6	4	378	1450	NA	52	6	378	90	NA
	C	8	6	457	360	90	118	4	457	90	23
II	D	4	23	187	360	NA	27	4	387	90	NA
	E	7	90	367	1024	512	31	6	367	128	90
	F	7	45	202§	90	NA		0		0	NA
III	G	18	128	161	1450	64	40	4	161	64	23
	H	4	4	193	512	360	17	6	193	45	16
	I	18	45	370	512	90	24	6	370	256	32

NA = Serum not available § = Patient followed for 202 days only

available three years and a half later, have at this time titres of 11 and 23 respectively.

In GPS free titrations, only patient B shows antibody from day 52 when an even gradual rise begins. In patients A and C, neutralizing antibody appears even later and reaches approximately the same level in the 6 and 4 months specimens which for these patients are the next available. In all three patients the antibody curve follows at a lower level the titres obtained in the GPS supplemented sera. In the GPS free serum of patient A drawn three years and a half after illness, antibody can no longer be demonstrated.

Group II includes patients D, E and F, whose antibody pattern shows a rapid and abrupt rise in the titre of the GPS supplemented sera within the first week of illness. In the two patients in whom antibody is demonstrable in GPS free titrations, the antibody appears after approximately three weeks. Maximal titres appear 6 to 12 months after illness.

Titrations of the GPS supplemented sera show antibody titres of 23 to 90 in the blood specimens obtained during the first week after rash. At this time the titres appear to be reaching a plateau level where they persist unchanged for at least 1 to 2 months, after which period an increase is seen with peak titres of 90 to 1024 appearing 6 to 12 months after onset of illness. In the last available blood specimen from patient F, collected approximately 7 months after rash, the titre is 90. Patient E, from whom a blood specimen is available three years and a half later, has at this time a titre of 512.

In group II the GPS free serum specimens do not show appearance

of antibody until the end of the first month where titres of 4 and 6 are obtained in sera from patients D and L. In these patients titres are gradually increasing until peak titres are reached at the same time as that found for the GPS supplemented sera. In patient F antibody is not demonstrable in any GPS free titration throughout the observation period of seven months. The serum specimen obtained three years and a half later from patient E shows a hardly significant 2 fold decrease from the maximal titre in parallel with the findings in the GPS supplemented titration.

Group III comprises patients G, H and J. The antibody curves resemble those of group II with the exception that the initial rise in antibody titre in the GPS supplemented sera seem to appear more slowly in group III. The rather long interval of approximately 2 weeks between the first and second bleeding of the group III patients renders however an evaluation of this feature impossible.

In the GPS supplemented sera an antibody titre of 4 is found in serum from patient H on day 4, while antibody in the other two patients appears later. In all three patients a plateau level with titres of 45 to 128 is reached in the second blood specimen drawn 17 to 18 days after the rash. The titres persist at this level for at least 2 months and increase thereafter to maximal levels of 512 to 1450 6 to 12 months after onset of illness. The sera obtained three years and a half after illness show a decrease in titre from the maximal value down to or slightly above the plateau levels.

The antibody curves representing GPS free titrations follow a course similar to that of the group II curves.

DISCUSSION

The clinical diagnosis of rubella is often difficult due to atypical or inapparent manifestation of this disease (4) and serological methods for the demonstration of rubella infection are consequently desirable.

The work reported on here was initiated in 1964 with the objective of studying the development and persistence of neutralizing antibody to rubella virus in patients in order to work out a technique by which a reliable serological diagnosis of rubella might be established.

In accordance with the common procedure in virus laboratories (8) all the human sera collected were routinely heat inactivated at 56 C for 30 minutes. This procedure was later shown to reduce titres of neutralizing antibody to rubella virus (10, 11, 12). In a previous study (5) results were reported to be inconsistent when heated and unheated sera were tested simultaneously in rubella neutralization tests.

However, normal unheated GPS has been found capable of restoring the neutralizing capacity of heated human sera and also of enhancing the neutralizing effect of unheated sera (6, 14). Based on these find

ings the heated human sera in this study have been examined in the presence and absence of GPS

The appearance of rubella neutralizing antibody is generally assumed to occur within a few days of the onset of clinical disease increasing in titre until maximal levels usually from 16 to 256 are reached three to four weeks after rash The antibody is reported to persist unaltered in titre for many years probably indefinitely (1 2 3 12 15)

The present investigation confirms the rapid occurrence of rubella neutralizing antibody but contrary to previous findings our observations show that maximal titres may not be reached until 6 to 15 months after rash when titres from 90 to 1450 are encountered Since the late appearance of maximal titres is seen in all patients examined we have reason to believe that this applies to rubella patients in general After having reached the peak on the antibody curve titres tend to decrease to levels roughly approximating the titres observed during the first weeks of convalescence The level of these late titres are in agreement with the findings obtained by several investigators (3 9 15) including ourselves 7) who in immune adults have observed rubella neutralizing antibody titres of roughly the same magnitude as those seen in the patients included in this study after a period of three years and a half

From the antibody curves illustrated in Fig 1 it will be seen that titres of rubella neutralizing antibody in heat inactivated sera are greatly dependent on time of collection and also on the presence or absence of GPS Antibody in sera collected during the first months after illness may if titrated without GPS either not be demonstrable at all *i e* in the present study in a dilution of 1 4 or it may appear only in low titre In late serum specimens this condition appears to be less pronounced The serum of one patient however always required GPS in another patient a decrease in antibody titre below 4 was observed in GPS free titration by the end of the observation period covering three years and a half

Previous reports have indicated differences in antibody levels dependent on the form in which the sera were tested In a study on the serological diagnosis of rubella *Plotkin* (13) found neutralization titres in unheated sera to be higher than titres obtained in heated sera whether these were examined with or without the addition of normal rabbit serum He reports titres from 16 to 512 in unheated sera 3 to 4 weeks after rash *Parkman et al* (12) observed enhancement of neutralizing antibody when heated sera were supplemented with rabbit serum and found maximal titres of 64 3 to 4 weeks after illness while *Green et al* (2) using heated sera without addition of normal animal serum after a similar period of time encountered rather low maximal titres of 8 to 16

Antibody titres obtained in different laboratories may vary because of heterogeneity of test systems employed Apart from such variation the observations in the present study of differences in the GPS depen

dency of rubella antibody in early and late serum specimens may however offer an explanation of some of the apparent discrepancies in the antibody titres reported by different investigators

When titrations of sera are performed in the presence of GPS antibody is demonstrable in all specimens except as could be expected some of the early sera collected within the first week of illness As a general rule all GPS supplemented sera show higher antibody titres than the corresponding GPS free titrations The rather unexpected finding of maximal titres as high as 1450 in two patients may be due to the antibody potentiating influence of guinea pig serum which in our experience has a more pronounced effect than the more widely used rabbit serum (6)

A comparative examination of rubella neutralizing antibody in heated and unheated sera titrated with and without GPS has unfortunately not been possible with the material available for the present study since as mentioned routine heat inactivation of a majority of the serum samples had already been accomplished at the time when the adverse effect of this treatment became generally known

A serological diagnosis in virus diseases is often feasible by examination of 2 serum specimens collected 2-3 weeks apart early in the illness The present observations show that in order to demonstrate a 4 fold or higher rise in rubella neutralizing antibody titre in paired sera it is necessary to obtain the first serum sample at the appearance of rash or during the first days of illness If the first serum is collected after the first appearance of antibody a rather long interval between the two serum specimens is required before a significant difference in rubella neutralizing antibody titre can be demonstrated In cases where therapeutic abortion is contemplated a major extention of the period between collection of the paired sera is obviously excluded and it is hoped that an application of haemagglutination inhibition tests and complement fixation tests on the serial serum samples now investigated in neutralization tests may yield information useful for the serological diagnosis of rubella Although it should be stressed that the observations are too few to allow generalizations the results of the present study indicate however that it may be possible to estimate whether a neutralizing antibody titre in a single serum sample derives from a recent or a past infection Absence of antibody in the GPS free titration and demonstrable antibody in the GPS supplemented titration suggest a recent infection i e within the first months of rash although it appears from Fig 1 that this reaction may also occasionally be found at a later stage Simultaneous demonstration of neutralizing antibody in the GPS free and the GPS supplemented serum specimen may be possible from a few weeks after rash

Relatively high titres in this study titres above 512 may be indicative of the "peak titre period from 6 to 15 months after illness

Results of treatment with human serum and gamma globulin for

prevention of clinical rubella have been rather varying (2). Amongst several probable explanations of the variation in the protective value of gamma globulin *Schiff et al* (15) mention the difference in neutralizing antibody contents in several lots of gamma globulin assayed. They suggest that gamma globulin intended for prophylactic use be selected on the basis of a high titre of rubella neutralizing antibody. The finding in the present study that rubella patients develop maximal antibody titres as late as 6–15 months after illness suggests that material suitable for passive rubella immunization should be derived from blood donors 6 to 15 months after clinical rubella rather than from patients in the convalescent phase of the disease.

SUMMARY

Development and persistence of neutralizing antibody in human rubella infection have been studied in 9 patients bled at intervals from onset of illness over periods covering from one to three years and a half. All sera were heat inactivated and examined with and without the addition of normal guinea pig serum (GPS).

In GPS supplemented titrations rubella neutralizing antibody appear within a few days after onset of rash either with an abrupt or a more gradual rise in titre. Extraordinary high maximal titres up to 1450 are unexpectedly found as late as 6 to 15 months after infection. A decrease in titre is seen later although neutralizing antibody still persist at comparatively high levels at least three years and a half after illness.

In GPS free titrations, i.e. in heated patient sera without addition of GPS, the rubella neutralizing antibody titres are generally low and appear later than those found in the GPS supplemented titrations.

The results of the present study confirm the recently reported finding (6) that rubella neutralization in heated human sera is markedly dependent on the antibody potentiating factor in guinea pig serum. GPS appears to be necessary especially for the demonstration of early antibody while neutralizing antibody developed at a later date seems less GPS dependent.

REFERENCES

1. *Dudgeon J A*: Maternal Rubella and its Effect on the Foetus. Arch Dis Childh 42 110–125 1967.
2. *[illegible]en R H, Balsamo M R, Giles J P, Krugman S & Mirick G*: Studies of the Natural History and Prevention of Rubella. Amer J Dis Child 110 148–365 1965.
3. *Hutchinson D N & Thompson A M*: Continuous Rabbit Kidney Cell Cultures in the Diagnosis of Rubella Infections. Mon Bull Minist Hlth 24 38[illegible]–391 1965.
4. *[illegible] J S*: Rubella and Rubelliform Rash. Bact Rev 28 452–457 1964.
5. *[illegible]hay J*: Neutralization of Rubella Virus in a Rabbit Cornea Cell Line (SIRC). Acta path microbiol scandinav 67 153–159 1966.
6. *[illegible]hay J*: Rubella Virus Neutralization in Heated Sera. Acta path microbiol scandinav 73 275–[illegible] 1968.

7 *Leerhøy J* Neutralizing Antibody to Rubella Virus in Sera Collected in Denmark Danish med Bull In print

8 *Lennette E H* In Diagnostic Procedures for Viral and Rickettsial Diseases American Public Health Association N Y 3rd Ed p 44 1964

9 *Lundstrom R* *Svedmyr A* *Hagbard L* & *Kaijser K* Rubella Immunity as Related to Age and History of Overt Disease Acta Paed Scand 56 279-285 1967

10 *McCarthy K* *Taylor Robinson C H* & *Pillinger S E* Isolation of Rubella Virus from Cases in Britain Lancet II 593-598 1963

11 *Neva F A* & *Weller T H* Rubella Interferon and Factors Influencing the Indirect Neutralization Test for Rubella Virus Antibody J Immunol *93* 466-473 1964

12 *Parkman P D* *Mundon F K* *McCown J M* & *Buescher E L* Studies of Rubella II Neutralization of the Virus J Immunol 93 608-617 1964

13 *Plotkin S A* Virological Assistance in the Management of German Measles in Pregnancy J A M A *190* 265 268 1964

14 *Rawls W E* *Desmyter L* & *Melnick J L* Rubella Virus Neutralization by Plaque Reduction Proc Soc Exp Biol Med *124* 167-172 1967

15 *Schiff G M* *Sever J L* & *Huebner R J* Rubella Virus Neutralizing Antibody in Commercial Gamma Globulin Science *142* 58-60 1963

Acta path microbiol scandinav 74 85 92 1968

The Departments of Infectious Diseases Roslagstulls Hospital Stockholm and University Hospital Uppsala and the Department of Medicine II Veterinary College Stockholm

INFESTATION WITH DICROCOELIUM DENDRITICUM – THE SMALL LIVER FLUKE – IN ANIMALS AND HUMAN INDIVIDUALS IN SWEDEN

By

Elias Bengtsson Lars Hassler Paul Holtenius
Folke Nordbring and Gunnar Thorén

Received 26 ii 68

The small liver fluke has a cosmopolitan distribution but its occurrence has been reported mainly from the Northern hemisphere (3 6 8 11 13 14 23) It is well known also in Sweden (6 8 11) Infestation of cattle and sheep with the fluke *distomiasis* is responsible for substantial economic losses due to impaired milk produktion low meat quality and discarding of livers at slaughter Human infestations have been described mostly from Mediterranean and East European countries but seem to be sparse and often questionable or difficult to prove (2 5 17 20) Egg passers are all the more common The eggs have no pathogenetic importance but create a common diagnostic error in parasitological and clinical routine work

Very few reports of *Dicrocoelium dendriticum* findings originate from Africa In 1948 Roche in Nigeria identified a fluke from the liver in a viscerotomy section which he thought was *D dendriticum* (16) *Wolfe* (22) recently suggested that the frequency might be very high even there and *Odei* (22) found in Upper Volta and Mali that 58 per cent of cattle gall bladders examined in abattoirs from 1962 to 1964 contained what was identified as adult flukes or eggs of *D hospes* This species of *Dicrocoelium* was also observed by *Wolfe* in two jaundiced and three other patients The findings were judged as spurious infections The life cycle and pathogenicity of *D hospes* are considered to be the same as those of *D dendriticum*

In some parts of Sweden a high infestation rate of *D dendriticum* has been observed in cattle and sheep but so far any human cases have not been described The purpose of this paper is to summarize the veterinary reports on dicrocoeliosis and to report the first findings of eggs or infestation in man in Sweden An investigation of the frequency

of the disease in cattle in the same territory in Sweden for eight consecutive years is also presented

Life Cycle of the Parasite

The parasite has a rather unique life cycle with three hosts (7 10) Eggs from the definite animal host (mainly sheep) develop to a cercaria stage in snails (mostly *Helicella* and *Cionella* species) On wet days following long sunny days the larvae leave the snails in slimeballs (10) that are infective for ants of Formiaspecies in which metacercariae (0 5-1 0 mm long) develop

In case of affection of the pharyngeal ganglions the ant experiences an irresistible impulse to climb up on blades of grass or other herbs and clutch tightly there instead of setting off to the hill like the healthy ant There is a spasm of the jaw *musculature which renders it impossible for the ant to loosen its grip When the* temperature rises again the muscular spasm vanishes and the affected ants join their colony and behave like the healthy ones Cattle sheep and perhaps human beings eating herbs on which there are affected ants may be infected by metacercariae which via ductus choledochus migrate to the bile ducts and to the liver

In the definite host growth of the parasite is completed in seven weeks and another four weeks are needed until egg laying begins

In the liver of sheep infected with *D dendriticus* Vershinin observed incrustation with calcium salts in necrotic portions of the interlobular connective tissue and in the intima of the blood vessels There was also granular dystrophy of the parenchyma with simultaneous proliferation of liver cells and occasional thrombosis of blood vessels

The adult worm is 8-10 mm long and 1 5-2 5 mm broad The eggs are similar to eggs of *Heterophyes* species but differ from them in having a thicker shell and brownish granular content (Fig 1)

The developmental cycles of the intermediate hosts the snail and the ant are both equally essential In endemic areas the eggs produced by the adult worm in

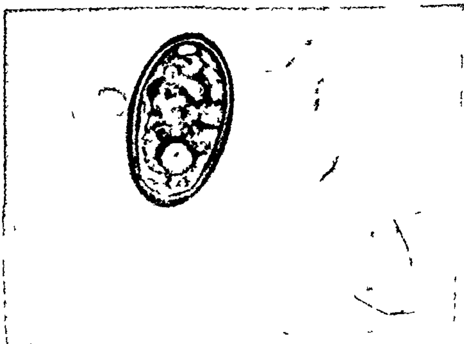


Fig 1

Egg of *Dicrocoelium dendriticum* in case no 1

the liver and the bile ducts of the animal host probably often form an invisible flavouring in the liver dishes we eat The eggs pass the gastro intestinal tract unnoticed But depending on the method of dispo al of faecal matter containing eggs snails may be infected and in such case open the way to infestation of animals or man to the extent that human beings consume infected ants

Previous Veterinary Investigations in Sweden

In *Hassler*'s total material of 2 246 cows distomiasis was caused by the small liver fluke in more than 90 per cent

Pathological investigations revealed a more or less severe chronic cholangitis mostly with a moderate or marked thickening of the walls On cross sections the bile ducts appeared as white demarcated circles from which bile and parasites welled out The cholangitis was purulent in approximately 10 per cent of all cases

Hassler noted that the consistency of an infected liver was often firmer than that of a liver free from parasites, more pronouncedly so in old animals Liver cirrhosis was rare Liver cysts were frequently observed many of them probably retention cysts subsequent to the infestation Infestation with *D dendriticum* in domestic animals does not give rise to manifest clinical symptoms

Immunity to the parasites has not been demonstrated

MATERIAL AND METHODS

Human Beings

The investigation on human subjects were performed during the last two years in the Parasitological Laboratory Roslagstulls Hospital Stockholm Approximately 8 000 faecal specimens from 4 000 patients have been investigated with regard to parasites almo t all of the patients ha l returned home from tropical regions most of them for a routine health control The fac al specimens have been examined micros opically according to *Ridley Hau good*'s concentration procedure (15)

Eggs of *D dendriticum* were demonstrated microscopically in faecal specimens from 15 patients treated in the Departments of Infectious Diseases in Stockholm and Uppsala Suspicions of other infest tions than dicrocoeliosis had primarily been raised in all instance and all patients except two had been abroad recently (Parasitological investigations are seldom performed unless the patient is a traveller) One patient had been in Chorea one in Nigeria two in Tanzania and nine in various Mediterranean countries

Animals

The stud es of the frequency of di croco l osis in cattle were performed during the years 1959 1986 within an area in the provinces of Östergotland and Småland. Altogether 2?1 ?90 head of cattle were examined at slaughter as described by Hassler in 1963 (8)

The cattle were divided into two groups young animals (up to 3 years of age) and older animals (cows) The degree of infestation among younger and older animals could thus be compared and any differences in frequency from year to year could be analysed in relation to possible changes in age distribution of the animal population as stigated

In ord r t det rmine whether different prerequisites of life for the intermediate hosts have an effect on the infestation rate of the definite host the animals were divided int two oth r groups those deriving from farms in forest districts being compared with those from farms on the plain

Collection and analysis of the materi l was carried out by Mr T Sollind Director of Östergötlands Andel slakteriforening Linköping

RESULTS

The occurrence of *D dendriticum* in the investigated animal population is shown in Table 1

The percentage of affected cattle within all groups did not change appreciably during the first five years of the study period. From the years 1963 to 1964 and 1964 to 1965 however a marked decrease in frequency of infestation was observed again within all groups. A slight increase appeared from 1965 to 1966. The incidence of disease in cows was about three times as high as in young cattle, three times as high in cattle younger or older from forest districts as in corresponding categories from the plain

TABLE 1
The Occurrence of Dicrocoelium dendriticum *in Swedish Cattle*

	Plains				Forest district			
	Young cattle		Cows		Young cattle		Cows	
Year	No of animals investigated	% affected	No of animals investigated	% affected	No of animals investigated	% affected	No of animals investigated	% affected
1959	3906	2.9	3712	7.4	9954	5.4	8395	20.6
1960	4729	2.3	3726	7.0	10580	5.0	7043	23.3
1961	4512	3.2	3418	7.3	9943	5.6	6511	23.5
1962	6602	2.7	3814	9.0	14803	7.2	7809	26.0
1963	7639	3.1	4425	9.5	16829	6.5	8421	23.6
1964	6883	1.4	4034	5.2	15530	3.4	8288	16.1
1965	6966	0.8	3731	4.4	15416	1.9	7004	13.3
1966	7963	1.1	4050	5.5	17235	2.3	7404	14.2

In the majority of the human cases eggs were found only in one of a series of faecal specimens. Epidemiological analysis including food consumption disclosed that 12 out of the 15 patients had eaten liver dishes (liver roasted whole, liver sausage, liver stew) one to four days before the sample was obtained. It is thus probable that the patients had pseudo infestations of *D dendriticum* and in most cases other diseases were revealed. In some cases any definite diagnosis could not be secured. One patient had a weakly positive serological test for amoebiasis in connection with a sligth blood eosinophilia and enlargement of the liver, another had leucocytosis, increased serum transaminase and diastase levels and a positive Schilling test and a third had a malabsorption syndrome

In two cases the disease pattern suggested rather dicrocoeliosis. The case histories were as follow

Case No 1 A 43 year old Swede was taken ill in a village near Barcelona where he had been living under primitive conditions The patient developed intense diarrhoea and a feeling of discomfort below the right costal margin and he was admitted after a three month period of symptoms Eggs of *D dendriticum* were found in faecal samples collected over a two day period In one sample there were also eggs of *Ascaris lumbricoides* Other pertinent findings were a mild blood eosinophilia (8 per cent) elevated serum cholesterol (3[illegible]-390 mg/100 ml) elevated beta globulin fraction on electrophoresis of serum a slight increase in bromsulphalein dye retention impaired xylose excretion and a flat glucose tolerance curve By roentgen graphs a barium meal was seen to pass rapidly through the intestines

The results might be in agreement with an obstructing process of the bile ducts and with intestinal malabsorption *e g* abnormalities that might be consistent with dicrocoeliosis Analysis of the food history disclosed that he had eaten large quantities of vegetables and salad which were not cooked or cleaned He had been particularly delighted in a dish called "paillia consisting of rice and snails

Case No 2 A 24 year-old female negro from Nigeria who previously had delighted in eating flying ants fried or cooked very lightly in oil The patient had also enjoyed eating snails washed with lime and lightly cooked The slime was difficult to remove when the snails were prepared She is also very fond of all kinds of liver dishes Constipation and stomachache are common complaints in her home district The patient herself had suffered from recurring abdominal pain and constipation for a couple of years Radiographs of the gall bladder and the large intestines was normal and so were the liver function tests Appendectomy gave no relief nor did a course of piperazine for suspected ascariasis In the stools we found repeatedly *Strongyloides* larvae as well as *D dendriticum* eggs At a follow up one year later the patient had still some abdominal discomfort but we found no more parasites

The chances of contamination seem to have been considerable in this case and the symptoms of abdominal pain and constipation might be consistent with dicrocoeliosis

DISCUSSION

The veterinary studies have shown that dicrocoeliosis is widespread in Sweden One of the districts investigated the coast north of Stockholm is one of the most popular holiday regions in the country Some risk of human infestation during camping seems probable According to *Krull & Mapes* (10) approximately 35 per cent of the ants in areas with a high rate of parasites are carriers of metacercariae Knowing the propensity of ants to turn up wherever there is something eatable it seems quite possible that they may contaminate food consumed in the open air

The clinical picture at previously reported human cases has been characterized by gastrointestinal symptoms Constipation has been common as well as diarrhoea and abdominal pains The liver has been enlarged and tender (17 18 23) In one case meningitis convulsions and paralysis were thought to be caused by a cerebral localization of the disease (19)

In the light of the widespread occurrence of dicrocoeliosis in Sweden and the veterinary importance of the disease it is surprising that so few cases of human infestation are found As a matter of fact we hesitate to diagnose any of our described cases as dicrocoeliosis The coexisting infestation with *Ascaris lumbricoides* and *Strongyloides stercoralis* might have contributed to some of the symptom patterns these very common parasites do however almost never give rise to liver function disturbances or signs of malabsorption The [illegible] have ex

RESULTS

The occurrence of *D dendriticum* in the investigated animal population is shown in Table 1

The percentage of affected cattle within all groups did not change appreciably during the first five years of the study period From the years 1963 to 1964 and 1964 to 1965 however a marked decrease in frequency of infestation was observed again within all groups A slight increase appeared from 1965 to 1966 The incidence of disease in cows was about three times as high as in young cattle, three times as high in cattle younger or older from forest districts as in corresponding categories from the plain

TABLE 1

The Occurrence of Dicrocoelium dendriticum in Swedish Cattle

	Plains				Forest district			
	Young cattle		Cows		Young cattle		Cows	
Year	No of animals investigated	% affected	No of animals investigated	% affected	No of animals investigated	% affected	No of animals investigated	% affected
1959	3906	2 9	3712	7 4	9954	5 4	8393	20 6
1960	4779	2 3	3726	7 6	10590	5 0	7043	73.3
1961	4512	3 2	3118	7 3	9913	5 6	6511	2 .5
1962	1602	2 7	3914	9 0	14803	7 2	7809	26 0
1963	/639	3 1	4495	9 5	1C929	6 5	84?1	1 6
1964	6883	1 4	4034	5 2	15530	3 4	8988	16 1
1965	6966	0 8	3731	4 4	15416	1 9	/004	13 3
1966	7913	1 1	4050	5 5	17235	2 3	7404	14?

In the majority of the human cases eggs were found only in one of a series of faecal specimens Epidemiological analysis including food consumption disclosed that 12 out of the 15 patients had eaten liver dishes (liver roasted whole liver sausage liver stew) one to four days before the sample was obtained It is thus probable that the patients had pseudo infestations of *D dendriticum* and in most cases other diseases were revealed In some cases any definite diagnosis could not be secured One patient had a weakly positive serological test for amoebiasis in connection with a slight blood eosinophilia and enlargement of the liver another had leucocytosis, increased serum transaminase and diastase levels and a positive *Schilling test* and a third had a malabsorption syndrome

In two cases, the disease pattern suggested rather dicrocoeliosis The case histories were as follow

rates from year to year within various age groups of domestic animals deriving from different geographical areas when the effect of combating procedures is to be appraised

The treatment of dicrocoeliosis has been an important and difficult problem in infected animals Emetin thymol furadin tetrachlorethylene and several other drugs were tried (12) without great success until *Lammler* (12) in detailed experiments with more than 800 derivatives found a preparation Hetolin synthetized by M Schorr Farbenwerke Hoechst A G which is highly active against *D dendriticum* This drug has been used in other series (4 9) with the same favourable results As far as we know it has not been used in human cases

SUMMARY

Dicrocoeliosis is a common infestation in Swedish cattle and sheep In some areas more than 50 per cent of the animals are affected Considerable variations in rates of infestation from year to year have been observed in animals from a particular district Older animals were affected to a much greater extent than younger The frequency of dicrocoeliosis was approximately three times as high in animals deriving from farms in the forest district as in those from farms on the plain

Eggs of *D dendriticum* were found in faecal specimens of 15 patients The majority of the patients had eaten meals consisting of liver dishes one to four days prior to sampling In an isolated case however there was a food history of ants and snails and in another case laboratory tests indicated an obstructive process of the bile ducts and intestinal malabsorption The difficulties in clinical diagnosis are discussed

REFERENCES

1 *B n x J Inmy I & Gledel J* Etude de la reaction de fixation du complément à l antig ne distomien chez le mouton Bull Soc Path Exot 5 83-87 1959
2 *B ghe van d n I & Deneche K* Dicrocoel um dendriticum (Fasciola lanceolata) chez l homme et les signes au Congo belge Ann Soc Belge Med Trop 18 509-514 1938
3 *Curran J A & Feng S T* A survey of human parasites of west central Shansi China Med J 44 891-904 1930
4 *von En gl K & Duw l D* Die Wi ksamkeit von Hetolin® auf den Dicrocoelium – Befall bei Schaf Rind und Pferd Dtsch Tier rztl Wchschr 70 377-391 1963
5 *Galli Val r B & Bornand M* La distomatose a *D crocoel um lan eolatum* chez l homme S hw Med Wchschr 12 (96) 614-615 1931
6 *H llund S Sallnäs T & Sefastsson S* Östergotlands andelsslakteriforenings rs edog rel e 19 9
7 *Hohorst W & Lammle C* Experimentelle Dicrocoeliose-Studien Ztschr f Tr penmed & Parasitologe 13 377-397 196
8 *H3 sl r I* Distomatos hos n tl reatur Nord Vet med 15 79-91 1963
9 *Ha sl r I H ll nsas F & Ljungbe g I* Provning av ett nytt medel mot lilla lev rflundran h s nothreatur Svensk Vet tidskr 17 441-443 19 5
10 *K ull W H & Mapes C R* Studies on the biol gy of *Dicro oel um dendriticum* The sec nd intermediate host. Cornell Vet 4? 2?? ??? ???

11 *Larsson G* Lilla leverflundran En exposé Nord Vet med 7 976–984 1955
12 *Lämmler G* Die experimentelle Chemotherapie der Trematoden – Infektionen und ihre Problematik Habilationsschrift Ztschr Tropenmed und Parasitol 15 95–123 1964
13 *Oelland F* Om utbredelse og hyppighet av den lille leverikte i Norge Norsk Veterinaertidskrift 47 2?–2? 96–100 1935
14 *Price E W & Kincheloe W D* The occurrence of *Dicrocoelium dendriticum* in the United States Amer Soc Parasitologists program and abstracts of the 17th ann meeting 27 Suppl 44 1941
15 *Ridley D S & Hawgood B C* Value of formol ether concentration of faecal cysts and ova J Clin Pathol 9 74–76 1956
16 *Roche I J I* Human Dicrocoelia is in Nigeria Trans Roy Soc Trop Med & Hyg 44 819–820 1950
17 *Scheid C & Mendheim H* Die Lanzettegelinfektion (Dicrocoeliasis) beim Menschen nebst Mitteilung eines neuen Falles Ztschr Tropenmed & Parasitolog 2 149–150 1950
18 *Sigalas R Leng Levy J David Chausse J Veaux R Wone Ch & Vail J* A propos d un cas de distomatose hepatobiliaire a *Dicrocoelium dendriticum* J Med de Bordeaux 34 595–599 1959
19 *Siguir F Feld M Pielle M Welti J J & Lumbroso P* Tribulations neurologiques d un jeune berger atteint de distomatose cerebrale a *Dicrocoelium lanceolatum* Bull Mem Soc Med Hop Paris 9 353–359 1959
20 *Skrjabin K I Schulz R Ed Sserbinoff P I & Smirnoff C G* Ergebnisse der Expedition zur Erforschung der Helminthosen der Kohlengrubearbeiter der Donjetzhohenkatte Zbl Bakt 112 454–458 1925
21 *Wasilijewa A I* Distomatosen der Leber bei Rindern in Turkestan (Fasciolasis und Dicrocoeliasis) Russ J Trop Med 5 no 1 1927 Cit H Vogel
22 *Wolfe M S* Spurious infection with *Dicrocoelium* Vosi in Ghana Amer J Trop Med & Hyg 15 180–182 1966
23 *Yenikomshian H H & Berberian D A* The occurrence and distribution of human helminthiasis in Syria and Lebanon Trans Roy Soc Trop Med & Hyg 27 425–435 1933

(Other references are available after request to the authors)

Acta path. microbiol. scandinav. 74: 93-100, 1968

Kaptein W. Wilhelmsen og Frues Bakteriologiske Institutt
University of Oslo, Norway

ISOLATION OF VARIANTS WITH INCREASED MUTABILITY FROM *NEISSERIA MENINGITIDIS*

By

KAARE JYSSUM and SIDSEL JYSSUM

Received 1.10.68

When hospital strains of *Escherichia coli* are screened for their mutation frequencies to streptomycin resistance and to auxotrophy, many strains are regularly found in which the mutation frequencies are increased (4, 3). This elevated mutability may be caused by mutator genes (1, 20, 18, 22, 21) but it may also be caused by some form of interaction between the chromosome and an element of episomic (or plastid) nature (3, 19). There is some indication that mutator genes as well as mutator factors of episomic nature are more frequently found among hospital strains of *E. coli* in 1966 than they were in 1958 and in 1960 (4, 3, *K. Jyssum*, unpublished results).

A mutability factor was also found in a *Neisseria meningitidis* auxotroph (11). This unstable variant which was fortuitously discovered during transformation of the auxotroph did not differ obviously from its parent strain except for its very high frequency of spontaneous mutation. Pathways for the biosynthesis of amino acids, vitamins, metabolization of sugars and resistance to antibiotics were all affected. It was assumed that the mutator property was the result of a spontaneous mutation in the actual strain, and the origin of the mutator strain thus more or less analogous to that of the Treffers factor (20) or the Harvard factor (1) in *E. coli*.

It has been the purpose of the present work to find out whether variants with increased mutation rates appear in populations of *N. meningitidis* with sufficient frequency to permit the isolation of a collection of mutator strains. Furthermore, it has been the intention to study the nature of the genetic lability in the mutator strains thus found with the hope of finding mutator factors with mechanisms of action different from those previously described from other microorganisms.

MATERIALS AND METHODS

St[illegible] The organisms which were screened for increased mutation frequencies were [illegible]lated from the cerebrospinal fluid of patients in Norway. Some of the strains have [illegible] ed in previous studies on the metabolism and genetics of meningococci (9, 10).

Genetic studies were performed with the following mutants obtained from the wild type strain M1 of Group B after ultraviolet light irradiation or nitrous acid treatment of cells (5) M1–18 his gly cp M1–8 his arg cp M1–5 his homoser cp M1–48 his cys cp M1 6 his pro cp Genetic competence is indicated by the symbol cp and incompetence which does not revert to competence by cp (7) The str-r marker was a single step high level resistant mutant (5)

Preparation of DNA Samples of DNA were obtained as previously described (5) In some experiments DNA was prepared by a phenol extraction procedure (13)

Media Blood agar plates or Heart Infusion Broth (Hib DIFCO) agar plates were used as solid complete media Fluid complete medium was Brain Heart Infusion Broth (BH Difco) Sugar markers were examined on Hib agar plates containing 2 6 ml per 1000 ml of a saturated solution of phenolred and the appropriate sugar in the concentration 1 per cent The basal media were those previously described (6)

Transformation The transformation assay followed the methods described previously (5)

Demonstration of genetic lability Genetic lability was scored by virtue of the high frequency in the population of mutants resistant to streptomycin Cultures of the N meningitidis strains were plated on complete media containing 100 μg streptomycin per ml (4) The mutation rates were usually determined from the number of tubes which contained no resistant variants (17 4) The proportion of auxotrophs of all variants in bacterial populations was determined by spreading on complete agar media and scoring single colonies on basal media either by streaking or by use of the velvet replica plating technique

Enzyme assays The assay of G-6-P dehydrogenase and glucokinase followed the procedures previously used (8) Protein was determined by a biuret method (10)

RESULTS

Search for Strains with Genetic Lability

Among 56 strains of N meningitidis which were scored for any unusual number of streptomycin resistant organisms by the plate method none was found which gave a high number of resistant colonies These strains had no unusually high frequency of auxotrophic mutation

Since it is known that mutator genes (or alleles) may arise in E coli strains with normal mutation rate probably by mutation (20 1) and since we had found one mutator strain in N meningitidis during a transformation experiment (11) it was considered likely that similar mutable variants could regularly be found in populations of meningococci provided an adequate selective procedure was established

The auxotroph M1 6 his pro cp is a double mutant previously used in numerous genetic experiments (9 10) The reversion frequencies of the nutritional markers his and pro in this strain are of the dimensions 4×10^{-11} and $< 2 \times 10^{-12}$ respectively The mutation rate to str r is $< 2 \times 10^{-11}$ It was assumed that a mutational event leading to the origin of a mutator allele in this strain could result in a lability of the biochemical markers Hence numerous spontaneous revertants to pro as well as to his were simultaneously scored for a high mutation frequency to str r and for any increased spontaneous auxotrophic mutation Among 3692 revertants to pro four were found which were sufficiently different from the typical parent strain to merit further investigation Mutation frequencies among 3105 revertants to his were

not considered different from those of the normal *N meningitidis* strain

Similar experiments have been performed with other nutritional mutants of the *N meningitidis* strain M1 It appears that mutator variants may be obtained by this procedure from most auxotrophs provided that the nutritional marker used is sufficiently stable *i e* with a reversion frequency at least less than 1×10^{-12}

Characteristics of Four Variants with Increased Mutability

The general characteristics of the four unstable variants isolated from the auxotroph M1 6 his pro cp have been successively examined along the same lines The four isolates seem to fall into the same class according to the criteria used Since no significant difference could be found this description is confined to the first one examined the mutant F9 his mut The mutator property has been indicated by the symbol mut

The unstable variant F9 and its various spontaneously occurring auxotrophs exhibited a considerable proportion of undersized surface colonies when streaked on complete media This may be due to the inability of various mutants to synthesize or take up substances In fluid media all derivatives carrying the mutator property showed a retardation of the growth rate This was observed during growth on basal media as well as on complete medium (Fig 1)

The total per cent of auxotrophs in cultures from blood agar plates ranged from 3 per cent to 8 per cent in fifteen determinations while the normal parent strain has less than 0 005 per cent if the same technique is used Requirements for a variety of amino acids (trc arg try gly his) and vitamins (nicotinamide pantothenic acid) were observed In many instances the growth requirements of the auxotrophs could not be identified by the usual auxanographic procedures (6 15) The nutritional requirements which were identified were in general single but the instability was seen to persist after a first mutation so that double auxotrophs arise frequently

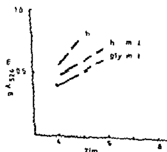


Fig 1

Retardation of the growth rate in auxotrophs carrying a mutator factor when grown in complete media

TABLE 1

Activities Corresponding to Glucokinase and G-6-P Dehydrogenase in Extracts from N meningitidis

Source of extract		Activity $A_{340} \times 10^3/min/0.1$ mg N		
Genotype	Adapted to	G-6-P Dehydrogenase NADP	NAD	Glucokinase NADP
Wild	Blood agar	268	108	57
Wild	Basal agar	273	111	103
his pro cp	Blood agar	312	128	180
his glc/mal mut cp		146	52	0
glc/mal cp		118	41	0

The metabolism of sugars was also affected. The mutants obtained fell into three classes. The most frequent class of mutants did neither metabolize glucose nor maltose. The negative property indicated by the symbol glc/mal seemed to be due to a mutation in a single locus (K. Jyssum & S. Jyssum, unpublished results). The lesion is probably a mutation in the hexokinase synthesizing system (Table 1). The other classes of mutants were glc+mal and glc mal. The last type of mutants was very rare in this mutator strain.

The mutation frequency str s to str r is also high in the labile variant. The mutation rate, i.e. the probability per replication act of that particular mutation, was estimated to 5.7×10^{-8} while the parent strain gave a rate less than 2×10^{-1} by the same technique (17, 4). It was observed that the mutator variant gave more than 98 per cent str r mutants. This is in contrast to the parent strain in which more than 80 per cent of the spontaneous mutants are streptomycin dependent. In the ancestor strain MI-12 his, the screening concentration of 100 µg per ml streptomycin results in 93.5 per cent dependent variants (16). The mutator strain does not carry the "normal" str r allele which is considered recessive (14) since DNA from the mutator strain does not transform a cp receptor strain from str s to str r. Also the mutator factor does not confer low resistance or protection against the streptomycin killing on meningococci like the episome-like factor in E. coli (2).

The stability of the mutator property was also examined on a more limited scale by testing colonies from single streptomycin sensitive units for the presence of appreciable numbers of resistant cells. Among more than 5000 colonies examined by this technique none had lost the mutation factor. Treatment with acridines (7) did not result in a loss of the mutator property.

Transformation of the Mutator Factor

In order to be able to study the genetic nature of the mutator property it has to be introduced into recipients so as to occur in combi-

nation with various genetic markers As no direct selection for the mutator property can be made the recombinants arising by transformation with DNA from the mutator strain were selected as mutators by the effect of the mutator factor on various biochemical markers From Table 2 is seen that the mutator factor which is obviously present in the variant F9 his-mut cp could be transformed into various recipients by this technique

Experiments similar to those recorded in Table 2 were performed with DNA from the four variants with increased mutation frequencies In all instances the results indicated that the mutator properties could be transformed into mutator negative competent receptor strains

TABLE 2

Transformation of a Mutator Factor into some Auxotrophs of N meningitidis

Recipient	DNA	Selective system: Medium	Cols per ml	Scored mutators Per cent of selected marker
his pro cp	his mut	His	> 5 0 × 10	< 0 5
		Pro	1 5 × 10²	95
	his str r mut	Str	> 5 0 × 10	Not tested
		His	> 5 0 × 10	< 0 5
		Pro	6 0 × 10	90
his arg cp	his mut	His	> 5 0 × 10	< 0 5
		Arg	2 1 × 10	86
hi pro cp	"	His	79	98
	pro mut	Pro	> 1 0 × 10⁶	< 0 5
his homoser cp	his mut	His	> 5 0 × 10	< 0 5
		Tre + Met	6 1 × 10	92
his gly cp	his mut	His	> 5 0 × 10	< 0 5
		Gly	1 8 × 10	92
his cys cp	his mut	His	> 3 0 × 10	< 0 5
		Cys	100	65

The transformation system contained approximately 10⁸ units per ml of the receptor strain The DNA contained 50 μg per ml Transformed (or mutated) clones were counted on appropriately supplemented basal agar plates Individual isolates were scored as mutators by spreading on complete media which were transferred to similar plates with 200 μg streptomycin per ml after 4 h preincubation (16)

DISCUSSION

Genetic instability does not at present seem to be an important problem among hospital strains of N *meningitidis* in Norway Thus the situation with this microbe differs from that of *E coli* in which genetic instability may have some selective advantage in hospital milieu (4, 3) On the other hand these experiments indicate that abrupt changes may occur in N *meningitidis* strains leading to persistent genetic instability although the strains had mutation frequencies which were considered normal when isolated from the patient The unstable lines do not differ obviously from its parent except for a somewhat slower growth rate and in the frequency with which mutants arise The retardation may

be a reflection of the heterogeneity with regard to growth properties observed during surface growth. However it could also point to some defect in the metabolism which might open an approach to the identification of the gene product of the mutator factor (12).

The frequency with which genetic instability arises in populations of *N. meningitidis* is obviously sufficiently high to permit the isolation of a collection of mutator variants along the lines established in the experimental section. Although the four unstable strains isolated in this series of experiments do not show any significant difference according to the criteria actually tested, they may obviously represent widely different mechanisms of action and separate genotypes. It seems likely that such an isolation of a collection of mutator strains should yield a valuable material for a further elucidation of the genetic nature and mechanism of action of increased spontaneous mutation in bacteria.

If we assume that the mutator phenomenon represents more or less an increased frequency of normal spontaneous mutation, the types of mutants obtained become of some interest. We have described the three classes of mutator induced mutants with defects in the sugar metabolization. Among these the variants glc mal$^+$ and glc mal$^-$ were known from meningococci in their natural habitat before. But the class glc/mal was not known to exist when it first turned up as the most frequent class among mutator induced mutants. But only a short time later we isolated such a strain from the cerebrospinal fluid of a patient in the University Clinic (Strain B 8152/66) and a year later we received another strain with the same type of mutation (Strain 15459/67) from Dr. *A. Reyn*, Copenhagen. Thus it seems that a survey of the mutator induced mutants may provide material to forsee the variants that may eventually turn up among strains in their natural habitat.

Any genetic study of a mutator phenomenon requires that the property should be transferrable by a recombination system. The experiments performed with the present mutator strains indicate that the mutator properties in all instances may be transformed into mutator negative stocks with DNA from strains carrying the mutator character. It may be argued that the experimental system presented (Table 2) also selects new mutators in the population of recipient cells. However, since the control experiments with DNAase treatment of DNA were constantly negative, and keeping in mind the very small number of mutator variants actually found in any normal population (vide supra), the results may be taken to indicate a regular transfer of the mutator properties by transformation resulting in a successive mutation of the marker in question.

SUMMARY

Generalized genetic instability could not be demonstrated among 56 strains of *N. meningitidis* isolated from patients in Norway. A selective

procedure was established which demonstrated that abrupt changes may occur in N meningitidis strains with no more than normal mutation frequencies leading to persistent genetic instability Four mutator variants were isolated in one experiment It may be assumed that genetic instability arises in populations of N meningitidis with sufficient frequency to permit the isolation of a collection of mutator strains

In the unstable variants pathways for biosynthesis of amino acids vitamins metabolization of sugars and resistance to antibiotics were affected The unstable variants contained mutator factors which could be transformed into mutator negative strains of N meningitidis

REFERENCES

1 *Goldstein A & Smoot J S* A strain of *Escherichia coli* with an unusually high rate of auxotrophic mutation J Bact 70 588–595 1955

2 *Gundersen W B* Reduced streptomycin killing in *E coli* carrying the Mu factor in its extrachromosomal state Acta path microbiol scandinav 65 627–635 1965

3 *Gundersen W B Jyssum K & Lie S* Genetic instability with episome mediated transfer in *E coli* J Bact 83 616–623 1962

4 *Jyssum K* Observations of two types of genetic instability in *Escherichia coli* Acta path microbiol scand nav 48 113–120 1960

5 *Jyssum K* Polarity of chromosome replication in *Neisseria meningitidis* J Bact 90 1182–1187 1965

6 *Jyssum K* Isolation of auxotrophs from *Neisseria meningitidis* Acta path microbiol scandinav 63 435–444 1965

7 *Jyssum K* Genetic factors determining competence in transformation of *Neisseria meningitidis* 2 A reversible loss of competence Acta path microbi l scandinav 67 493–502 1966

8 *Jyssum K Borchgrevink B & Jyssum S* Glucose catabolism in *Neisseria meningitidis* 1 Glucose oxidation and intermediate reactions of the Embden Meyerhof pathway Acta path microbiol scandinav 53 71–83 1961

9 *Jyssum K & Joner P E* Phosphorylation coupled to the reduction of cytochrome c by hydroxylamine in extracts from *Neisseria meningitidis* Acta path microbiol scandinav 62 390–398 1964

10 *Jyssum K & Jyssum S* Variation in density and transformation potential in deoxyribonucleic acid from *Neisseria meningitidis* J Bact 90 1513–1519 1965

11 *Jyssum K & Jyssum S* A genetic locus responsible for high mutability in *Neisseria meningitidis* Acta path microbiol scandinav Suppl 187 52 1967

12 *Kirchner C E J & Rudden M J* Location of a mutator gene in *Salmonella typhimurium* by cotransduction J Bact 92 1453–1456 1966

13 *Kleinschmidt A K Kass S J Williams R C & Knight C A* Cyclic DNA of shope papilloma virus J molec Biol 13 749–756 1965

14 *Lederberg J* Streptomycin resistance a genetically recessive mutation J Bact 61 549–550 1951

15 *Lie S* Production of auxotrophic mutants of *Neisseria meningitidis* by nitrous acid Acta path microbi l scandinav 63 615–622 1965

16 *Lie S* Studies on the streptomycin resistance system of *Neisseria meningitidis* Acta path microbiol scandinav 63 623–635 1965

17 *Luria S E & Delbrück M* Mutations of bacteria from virus sensitivity to virus resistance Genetics 28 491–511 1943

18 *Miyake T* Mutator factor in *Salmonella typhimurium* Genetics 45 11–14 1960

19 *Taylor A L* Bacteriophage induced mutation in *Escherichia coli* Proc nat Acad Sci (Wash) 50 1043–1051 1963

20 *Treffers H P*, *Spinelli V* & *Belser N O* A factor (or mutator gene) influencing mutation rates in *Escherichia coli* Proc nat Acad Sci (Wash) 40 1064 1071 1954
21 *Yanofsky C*, *Cox E C* & *Horn V* The unusual mutagenic specificity of an *E coli* mutator gene Proc nat Acad Sci (Wash) 55 274–281 1966
22 *Zamenhof P J* A genetic locus responsible for generalized high mutability in *Escherichia coli* Proc nat Acad Sci (Wash) 56 845 852 1966

Acta path microbiol scandinav 74 101-113 1968

The Institute of Clinical Bacteriology (Head Sten Winblad M D) The Department of Surgery (Head Helge Wulff M D) University of Lund General Hospital Malmö The Department of Infectious Diseases (Head Arne Lofdahl M D) Central Hospital Karlskrona The Department of Infectious Diseases (Head Sven Bilfrage M D) General Hospital Malmö Sweden

YERSINIA ENTEROCOLITICA IN PATIENTS WITH SYMPTOMS OF INFECTIOUS DISEASE

By

BIRGITTA NILÉHN BJÖRN SJÖSTRÖM KAREN DAMGAARD and CLAES KINDMARK

Received 1 iii 68

The occurrence of *Yersinia enterocolitica* in association with various diseases has been considered to argue for the pathogenicity of this micro-organism for human beings (*Hassig et al* 1949 *Carlsson et al* 1964 *Mollaret et al* 1964 *Wauters & Mollaret* 1965 *Winblad et al* 1966 (a b) *Graux & Wauters* 1966) The results of a recent investigation of the incidence of *Yersinia enterocolitica* in patients with acute abdominal symptoms (*Nilehn & Sjostrom* 1967b) suggest that the bacterium may sometimes be a causal factor of acute terminal ileitis and sometimes also of mesenteric lymphadenitis or other conditions simulating acute appendicitis The bacterium has however also been isolated from patients with diarrhoea erythema nodosum or other symptoms of acute infectious disease This paper describes the bacteriological findings and symptoms in such cases

MATERIAL AND METHODS

Clinical Material

The material consisted of 31 cases where *Yersinia enterocolitica* had been isolated from the faeces ([illegible] 1965 March 1967) Excerpts of these 31 cases are given in the appendix

Bacteriological Technique

Culture technique Faecal samples were cultured largely as described previously (*Nilehn & Sjostrom* 1967b) with the use of lactose sucrose urea agar (LSU agar) (*Juhlin & E[illegible]* 196[illegible]) slightly modified in using peptone special Orthana (A/S Orthana kemisk fabrik Copenhagen Denmark) instead of peptone Lelaf as a primary medium incubated at 2[illegible] C and 37 C and Rappaport broth (*Rappaport et al* 1956 1959) [illegible] C and 37 C 4[illegible] hours as a selective enrichment medium In a

---

We are indebted to Dr B Bollin Malmö J A Espensen Borgholm J Lindh Oskarshamn F Lindholm Växjö and B Nyström Karlshamn who kindly placed clinical data at our disposal

The investigation was supported by grants from the Medical Faculty of the University of Lund Sweden

few cases primary isolation was done on SS agar (Bacto SS agar B 74 Difco Laboratories Detroit Michigan) at 22 C

Bacteriological analysis *Yersinia enterocolitica* was identified and characterized with the methods described previously (*Nilehn & Sjöstrom* 1967b) Previously described tests were supplemented by investigation in oxidation/fermentation (O/F) media according to *Hugh & Leifson* (1953) for estimation of the activity of the strains on d xylose (E Merck AG Darmstadt Germany 8682) d glucose (Baker Chem Co Phillipsburg N J 1916) lactose (Baker Chem Co 2218) and maltose (Merck 5910) One per cent test substance was used final pH of the medium 7 0–7 1 As peptone Bacto casitone (Difco 0259-01) was used The medium was dispensed to tubes 10 × 150 mm 6 cm layer covered with aluminium caps Each strain was examined in tubes with and without 1 cm layer of paraffin covering the medium incubation at room temperature (22 C ± 2 C) and at 37 C ± 0 5 C The tests were performed at least in duplicate

Serological Investigation

Examination of the patients sera for agglutinins against O antigen preparations of *Y enterocolitica* (strain Winblad) according to the technique described previously (*Winblad et al* 1966b *Nilehn & Sjöstrom* 1967b) and determination of the O antigen type of the isolated *Yersinia enterocolitica* strains were performed by Professor *Sten Winblad*

RESULTS

Bacteriological Results

Three of the isolated 31 strains (strains 5674 582 and 1168) from cases 5 18 and 22 have been described previously (*Nilehn* 1967a) Most of the other strains appeared to be largely identical regarding previously tested biochemical and physiological properties One strain was however Voges Proskauer negative (case 8) and two (cases 14 26) differed regarding acid formation from d xylose (1 per cent) in liquid medium where an indicator shift was registered already after 3–5 days at both 22 C and 37 C In O/F media the strains showed further differences in the metabolism of d xylose and lactose (Table 1)

TABLE 1

Acid Formation from d Xylose and Lactose by 31 Human Strains of Yersinia enterocolitica *Tested in O/F Medium at 22 C and 37 C*

	Number of strains	22 C 1	2	37 C 1	2	Strains isolated from cases no
d xylose	2	1–2	1–2	1–2	1–2	14 26
	25	10	10–20	10–20	10 30	
	4	–	–	–		6 13 15 22
lactose	2	2	10	10	20	14 26
	23	20–30	20–30	20–30	20 30	
	6	–	30	20–30	20–30	2 6 10 12 23 29

The figures indicate the day on which indicator shift was registered The tubes were inspected after 1 2 3 5 10 20 and 30 days incubation

– = negative after 30 days
1 = reaction in tube without paraffin
2 = reaction in tube with paraffin
Acid formed slower than in tube 1

Thus 3 types of behaviour were observed in the metabolism of d xylose rapid acid formation (1–2 days) at both 37 C and 22 C (cases 14 26) late acid formation (10–30 days) at both temperatures and no acid reaction after incubation for 30 days (cases 6 13 15 22) As to lactose metabolism most of the strains produced acid slowly (20–30 days) at one or both incubation temperatures while 2 strains (cases 14 26) differed by their rapid oxidative formation of acid at 22 C but slow formation at 37 C None of the 31 strains showed formation of acid from lactose in liquid medium with 1 per cent peptone content during 30 days incubation All strains possessed β galactosidase activity more pronounced at 22 C than at 37 C

Acid was formed rapidly (1 day) from d glucose in O/F medium at both temperatures without any appreciable difference between tubes with and without paraffin As to maltose acid formation in O/F medium was likewise rapid (1–2 days) possibly somewhat slower at 37 C than at 22 C

The susceptibility of the 31 strains to antibiotics was largely the same as that of previously described human *Yersinia enterocolitica* strains (Mälehn 1967a Mälehn & Sjöström 1967b)

The 2 strains that differed from the others in rapid acid formation from d xylose and lactose (cases 14 26) were also found to differ in respect of O antigen type (Winblad 1967 unpublished observations)

Culture from the faecal samples from the 31 patients could not reveal any *Salmonella Shigella* or *Staph aureus*

Clinical and Serological Observations

The main symptoms and maximum agglutinin titres of the 31 patients with positive *Yersinia enterocolitica* cultures are summarized in Table 2 Twenty four of the patients had diarrhoea in 2 cases with bloody stools Eleven of the patients with diarrhoea had also had colic for which 2 had attended the surgical department because of suspected appendicitis In one case (case 1) the abdominal pain suggested biliary disease Roentgen examination of that patient revealed gallstone as a probable explanation of the acute symptoms During the last few years one patient (case 16) had been treated because of suspected ulcerative colitis

A body temperature of more than 38 C was recorded in 18 of the 31 patients In 2 cases (cases 7 8) temperature peaks of 39–40 C had been noted while in one (case 5) the temperature remained elevated for a long time

Vomiting or nausea occurred in only 4 cases Nonspecific muscular pain or joint pain had been noted in 3 and 4 cases respectively In 2 cases there had been signs of acute gonarthritis with reddening warmness and exudate Seven patients including 6 with symptoms of diarrhoea had also had upper respiratory tract infections

TABLE

Main Symptoms and Maximum Agglutinin Titre (Reciprocal Value) against O Antigen ... enterocolitica Had Been

Case no	Age (years)	Sex		Diarrhoea	Fever (≥ 38 °C)	Abdominal pain	Erythema nodosum	Arthralgia	Upper respiratory infection	Muscular pain	Vomitus/Nausea	Maximum agglutinin titre
1	80	F			+	+						160
2	[illegible]	F		+		+	+					2560
3	69	F			+		+					5120
4	68	F		+	+		+	+				1280
5	63	F		+	+	+		+		+		20480
6	63	F		+	+	+	+	+				5120
7	58		M	+	+					+	+	5120
8	55	F		+	+	+	+					5120
9	46	F			+		+			+		5120
10	44	F		+	+	+	+				+	1280
11	44	F		+	?							5120
12	40	F		+								1280
13	39	F				?	+	+				640
14	35		M	+		+			+			1280
15	31	F		?			+					–
16	26	F		+								160
17	26	F				?			+			320
18	25		M					+				1280
19	25	F		+		+						320
20	23	F		+	+	+						160
21	2?	F		+	+	+	+	+				5120
22	[illegible]		M	+	+	+			+			1280
23	16		M	+	+				+			1280
24	15	F		+	+				+			640
25	6		M	+	+	+						320
26	3		M	+	+							2560
27	2	F		+								10
28	2	F		+	+				+			?
29	2		M	+	+						+	?
30	11/12		M	+					+			?
31	4/12		M	+							+	?
Total	31	21	10	24	18	1	10	6	7	3	4	20

In one patient (case 17) the only abnormality found was an elevation of the E S R

Ten of the female patients had erythema nodosum In 6 cases the erythema had occurred after an acute attack of diarrhoea possibly also fever or joint pain in 2 after somewhat uncertain prodromal symptoms and in 2 as the initial symptom observed The interval between the onset of diarrhoea and the appearance of erythema had ranged from 7 to 16 days One of the patients had a positive antistreptolysin titre but culture of throat swabs was negative In the other cases clinical investigation revealed no other explanation of the erythema

...eparation of Yersinia enterocolitica *Human Type Thirty one Cases in Which Yersinia* ...*olated from the Stools*

Remarks
Cholelithiasis
Chest X ray normal
Chest X ray normal Antistreptolysin test and Mantoux negative
Chest X ray normal Antistreptolysin test and Mantoux negative
Chest X ray normal Antistreptolysin test Mantoux and Waaler Rose negative
Chest X ray normal Mantoux negative
Chest X ray normal Antistreptolysin test Antistaphylolysin test and Mantoux negative
Chest X ray normal Antistreptolysin test positive Mantoux negative
Chest X ray normal Antistreptolysin test and Mantoux negative
Chest X ray normal Pregnant m IV
Suspected ulcerative colitis
Acute gonarthritis
Chest X ray normal Antistreptolysin test negative Mantoux positive Pregnant m III
Chest X ray normal

The ages of the 31 patients ranged from 4 months to 80 years Seven of the patients were children below 10 years The patients with erythema nodosum were 22 to 77 years old

Agglutinins against an O antigen preparation of the type strain Winblad were demonstrated in all of the cases studied except one (case 14) Two patients (cases 26 and 31) showed low convalescent titres of 1 10 and 1 20 respectively In the other cases the maximum titres ranged from 1 160–20 480 Four patients were not examined serologically

Twenty two of the patients received chemotherapy Treatment with

penicillin in 3 cases before sampling did not prevent subsequent isolation of the bacterium from the stools. Tetracyclines had been used in 17 cases; in 10 cases studied the bacterium could not be demonstrated in samples received after tetracycline therapy had been started.

Most of the patients soon recovered but in some of them the symptoms were prolonged (cases 5, 8, 31). Nineteen patients followed up were symptomfree after 2 to 26 months.

No epidemiological connection between the 31 positive cases was found.

DISCUSSION

The strains of *Yersinia enterocolitica* investigated here did not differ appreciably in various diagnostic tests from strains of human origin described previously (*Nilehn* 1967a, *Nilehn & Sjostrom* 1967b). However, one strain differed from the others by its negative Voges-Proskauer's reaction and two strains differed by their O antigen composition (Winblad 1967).

When cultured in O/F media according to *Hugh & Leifson* (1953) minor dissimilarities among the strains in their metabolism of d-xylose and lactose could be distinguished. Thus, the 2 antigenically divergent strains formed acid rapidly from these two carbohydrates in O/F medium, similar to the behaviour observed among *Yersinia enterocolitica* strains isolated from animals, especially chinchilla (*Frederiksen* 1967, *Nilehn* 1967b). The activity on lactose of all but these 2 strains manifested itself by acid formation observed late or very late in O/F medium and not visible in liquid medium with 1 per cent peptone. Even if the present study does not warrant any conclusions about the significance of the metabolic dissimilarities found, the reproducibility of the results argues against the postulation that they might be due to investigation of occasional mutant clones.

The influence of the peptone concentration in investigations of acid formation from carbohydrates is well known (*Lowe & Edwards* 1957, *Schaffer* 1962). With a view to demonstrating small amounts of acid formed by the strains, the O/F medium of *Hugh & Leifson* (1953) was found more suited than a liquid test medium with a higher peptone concentration, not only in the metabolism of lactose but also of d-xylose and maltose.

Variation in acid formation capacity with the incubation temperature has previously been noted in *Yersinia enterocolitica* strains (*Nilehn* 1967a). The difference in acid formation from maltose at 22° C and at 37° C (*Nilehn* 1967a, b) was, however, less readily demonstrable in the O/F medium, probably because of the ability of this medium to detect even small amounts of acid formed.

Previous publications (*Carlsson et al.* 1964, *Mollaret & Destombes* 1964, *Winblad et al.* 1966, *Nilehn & Sjostrom* 1967a, b) suggest that *Yersinia enterocolitica* may sometimes cause conditions with symptoms

simulating appendicitis such as acute terminal ileitis or mesenteric lymphadenitis. Isolation of *Yersinia enterocolitica* from the present patients with symptoms of various acute infections particularly fever diarrhoea and erythema nodosum raises the question whether the bacterium may cause these symptoms. It is noteworthy that many of the patients had also had acute abdominal pain sometimes simulating acute appendicitis. Single reports on *Yersinia enterocolitica* as a possible pathogen in patients with fever and gastro intestinal symptoms of similar type have been published previously (*Wyler*/cit. *Mollaret et al* 1964. *Wauters & Mollaret* 1965. *Graux & Wauters* 1966). But the present clinical material does not warrant any definite conclusions concerning a possible relation between the presence of the bacterium and the symptoms. Numerous other aetiological possibilities in patients with non specific symptoms such as fever and diarrhoea make it difficult to judge the pathogenetic significance of the bacterium.

Not only tuberculosis but also several other conditions are believed to be of aetiological significance in the development of erythema nodosum. Of interest in this connection are reports of erythema nodosum in association with the occurrence of the closely related *Yersinia pseudotuberculosis* (*Morger* 1962. *Mollaret* 1962. *Kerjoncuf* 1967). *Mollaret & Destombes* (1964) reported mesenteric lymphadenitis in a patient who later developed erythema nodosum and a high agglutinin titre against *Yersinia enterocolitica*. A further case with mesenteric lymphadenitis, development of agglutinins, isolation of *Yersinia enterocolitica* from the appendix and erythema nodosum about one week after the onset of symptoms has been described by *Ahlehn & Sjostrom* (1967a, b). Agglutinins against *Yersinia enterocolitica* have also been demonstrated in sera from a large number of patients with erythema nodosum (*Winblad* 1967), which argues for a relation between the bacterium and this symptom. The clinical investigation of the 10 patients described here produced no certain evidence of any other cause of the erythema.

One of the patients from whom *Yersinia enterocolitica* had been isolated appeared healthy apart from a high E S R. The results of a previous investigation (*Ahlehn & Sjostrom* 1967b) indicate that the bacterium is not common in various controls such as apparently healthy adults or children, gynaecological patients or patients with traumatic injury.

The high incidence of high agglutinin titres against *Yersinia enterocolitica* in patients with positive cultures might argue for an aetiological significance of the bacterium. In those cases that could be followed with repeated serological analysis the development of the agglutinins suggests a relationship with the acute disease. In one case where no agglutinins could be demonstrated and in another where the titre was only low the strains isolated from the patients were not antigenically identical with the others or with the strain used as antigen for the

serological examinations. But in neither of these cases was it possible to make any test for agglutinins against the patients' own strains.

Analysis of the available historical data of the geographical and chronological distribution of the cases revealed no epidemiological relation between them. In one patient (case 22) *Yersinia enterocolitica* had been isolated previously in the same family; the possibly common source is, however, not known.

Though it cannot be concluded with certainty that *Yersinia enterocolitica* was responsible for the symptom complexes or single symptoms in the present cases, the relation between the clinical course, bacterial findings, development of antibodies, and in most cases lack of other explanations of the symptoms argue for the bacterium being of aetiological significance.

SUMMARY

Thirty one cases in which *Yersinia enterocolitica* had been isolated from faeces were examined serologically and clinically. Minor biochemical and/or serological differences between individual isolated strains were demonstrated. High antibody titres were observed in 25 of 27 patients studied.

Diarrhoea or other gastro-intestinal symptoms, usually associated with fever, were common. The material included 10 cases of erythema nodosum.

CASE REPORTS

Case 1. Woman aged 80. Alternating diarrhoea and constipation for one year. In Nov. 1966 admitted to hospital (Malmö) because of fever and diffuse abdominal pain. Max. temp. 38.6 C; body temperature became normal after 4 days. X-ray: Cholelithiasis. Culture of faeces one week after admission gave growth of *Y. enterocolitica*. The woman left hospital symptomfree after 14 days' treatment with oxitetracycline. Agglutinin titre against *Y. enterocolitica* 1 week after admission 1:160, 3 weeks later 1:10 and after a further week 1:10. Symptomfree at review 5 months later.

Case 2. Woman aged 77. Admitted to local hospital (Borgholm) in Nov. 1966 one week after acute onset of diarrhoea and dull abdominal pain. X-ray of colon and chest: normal appearances. Sixteen days after onset of symptoms erythema nodosum appeared. Culture of faeces 3 weeks after onset of symptoms gave growth of *Y. enterocolitica* and serological examination then showed an agglutinin titre of 1:2560. After treatment with oxitetracycline culture of faeces gave no growth of *Y. enterocolitica*. All symptoms disappeared after 2 weeks' treatment. Agglutinin titre 6 weeks after onset 1:160, after further 3 weeks 1:40. Symptomfree at review 2 months after onset.

Case 3. Woman aged 68. Admitted to hospital (Karlskrona) in Sep. 1966 because of fever and erythema nodosum which had appeared 1–2 weeks previously. Treated with penicillin for 10 days before admission. No known gastro-intestinal symptoms. Culture of faeces on admission gave growth of *Y. enterocolitica*. The woman became afebrile a few days after admission. She was treated with demethylchlortetracycline and the erythema soon disappeared. Chest X-ray: normal appearance. Antistreptolysin titre negative. Mantoux negative. Agglutinin titre against *Y. enterocolitica* on admission 1:80 and 1:5120, 1:160 and 1:40 after 2, 7 and 11 weeks.

Case 4. Woman aged 68. Fever and diarrhoea for about a week in Dec. 1966. Then mild back and hip pain and about 14 days after onset of initial symptoms erythema nodosum. Admitted to hospital (Karlskrona). Chest X-ray: normal appearance. Antistreptolysin titre negative. Mantoux negative. Culture of faeces on admission gave growth of *Y. enterocolitica*; agglutinin titre 1:1 80. Treated with de

methylchlortetracycline for 11 days. At review 3 months after onset the patient was symptomfree agglutinin titre 1 640

Case 5 Woman aged 63 Two weeks of malaise in Nov 1965 was followed by acute onset of chills acute abdominal pain and intense diarrhoea The diarrhoea and the fever disappeared after one week The temperature then again began to rise and the woman complained of muscular pain and swelling and pain of left knee On admission to hospital (Malmo) her temperature was 38 5 C Cultures of blood and joint fluid negative Chest X ray normal appearance Mantoux negative antistreptolysin test negative Waaler Rose negative Widal s test (agglutinins against O and H antigen preparations of S typhi S paratyphi A and B) negative Culture of faeces some days after admission gave abundant growth of Y *enterocolitica* No growth after treatment with oxitetracycline Agglutinin titre against Y *enterocolitica* on admission 1 1280 1 week after admission 1 20 480 5 months after admission 1 320 The patient was first treated with benzylpenicillin 1 million × 4 then with oxitetracycline 2 g per day and after a further 10 days treatment with chloramphenicol 1 g × 2 was started The fever (38 C 39 C) persisted for 3 weeks after which the patient was subfebrile for a further 2 weeks Six weeks after admission she was sent home in a fairly good condition She was readmitted 14 days later because of suspected venous thrombosis but was sent home 10 days later Afterwards she felt well apart from symptoms of arthrosis deformans Last follow up 26 months after onset revealed nothing remarkable Agglutinin titre then 1 160

Case 6 Woman aged 63 Onset in March 1967 with diarrhoea for 4 days abdominal tenderness but no fever One week later fever pain in the ankle hand and finger joints Eleven days after onset of the symptoms erythema nodosum appeared on the forearms and lower extremities The patient was admitted to hospital (Malmo) Culture of the faeces gave growth of Y *enterocolitica* Treatment with oxitetracycline was started Eight days later the patient was afebrile Chest X ray normal appearance Antistreptolysin test negative Waaler Rose negative Sent home symptomfree after 3 weeks Agglutinin titre against Y *enterocolitica* on admission 1 5120 2 months after onset 1 320 Review 3 months later showed nothing remarkable

Case 7 Man aged 58 Previously healthy Acute onset in Nov 1966 with nausea and fever Two days later he had high grade fever and diarrhoea Afebrile after one week After a further day or so his body temperature again began to rise and he complained of general muscle pain Admitted to hospital (Malmo) where culture of the faeces gave abundant growth of Y *enterocolitica* Treated with oxitetracycline and symptoms promptly disappeared Sent home 10 days later Review 2 months later revealed nothing remarkable Agglutinin titre against Y *enterocolitica* on admission 1 5120 on discharge 1 2560 1 month after onset of symptoms 1 1280 5 months later 1 80

Case 8 Woman aged 57 In Oct. 1966 onset of fever 38–38 5 C diffuse epigastric pain and 2 days later diarrhoea This was followed by chills and fever peaks of 38 5–40 C for a week Fourteen days after onset diarrhoea returned and erythema nodosum appeared Admitted to hospital (Karlskrona) Body temperature on admission 38 2 C Culture of the faeces on admission gave growth of Y *enterocolitica* After 11 days treatment with demethylchlortetracycline body temperature was again normal Mantoux negative Chest X ray normal appearance Agglutinin titre against Y *enterocolitica* on admission 1 5120 after 3 weeks 1 2560 after 6 weeks 1 1280 and after 3½ months 1 320 Review 5 months after onset revealed nothing remarkable

Case 9 Woman aged 46 Previously healthy In Nov 1966 she developed erythema nodosum on the right lower extremity and after a few days malaise subfebrile body temperature and diffuse generalized pain On admission to hospital (Karlskrona) her body temperature was 38 2 C Mantoux negative Microscopy for tubercle bacilli in sputum negative Chest X ray normal appearance Test for antistreptolysin and antistaphylysin negative EKG normal Culture of faeces gave growth of Y *enterocolitica* The patient was treated with demethylchlortetracycline during which body temperature gradually returned towards normal Afebrile after 3 weeks She left hospital symptomfree after 4 weeks Agglutinin titre against Y *enterocolitica* on admission 1 1280 14 days later 1 1280 one month later 1 640 3 months after onset 1 80 At review 4 months later the patient felt well

Case 10 Woman aged 44 Previously healthy In Nov 1966 nausea abdominal pain borborygmus and mild diarrhoea Body temperature up to 38 C for 3–4 days

One week after onset of symptoms erythema nodosum appeared on lower extremities The patient was then admitted to hospital (Vaxjo) Body temperature then 38 C, Mantoux negative Chest X ray normal appearance Culture of throat swabs showed nothing abnormal Antistreptolys in titre 210 IL Culture of faeces immediately after admission gave growth of Y enterocolitica The woman was sent home symptom free after one week No known persistent symptoms Agglutinin titre against Y enterocolitica on admission 1 160 3 weeks later 1 5120 ? months after admission 1 2560

Case 11 Woman aged 44 Diarrhoea for 10 days after an episode of fever in Aug 1966 No treatment Culture of faecal samples after the symptoms had almost disappeared gave growth of Y nterocolitica Agglutinin titre against Y enterocolitica about 14 days after onset 1 160 one month later 1 1280

Case 12 Woman aged 40 Diarrhoea for about a week in Nov 1966 Sought advice at outpatient department Karlskrona hospital Culture of faeces gave growth of Y enterocolitica agglutinin titre 1 640 The patient was treated with tetracycline She soon felt well and investigation revealed nothing abnormal Review one month later showed nothing remarkable Agglutinin titre then 1 160

Case 13 Woman aged 39 The history included repeated attacks of tonsillitis last time 2 years before present disease which started in April 1966 with fatigue stabbing pain radiating from the lumbar region of the back forwardly towards the abdomen Two weeks later erythema nodosum appeared on the lower extremities in association with pain in the knees elbows and ankles She was admitted to hospital (Karlskrona) about one month after the onset of the initial symptoms The erythema then less intense Mantoux negative Antistreptolysin test negative Chest X ray normal appearance Culture of faeces gave growth of Y enterocolitica agglutinin titre 1 1280 two months and a half after onset 1 320 During her stay in hospital she was treated with tetracycline Review two months and a half after onset revealed nothing remarkable

Case 14 Male aged 35 Onset of mild respiratory tract symptom and subfebrility in Aug 1966 One week later alternating diarrhoea and constipation About 5 weeks after the onset of the initial symptoms diarrhoea recurred with intermittent abdominal pain in the right lower quadrant Admitted to hospital (Malmo) On admission he was afebrile and showed slight abdominal tenderness most accentuated over MacBurneys point Cluture gave growth of Y enterocolitica Treated with oxytetracycline Serological examination of blood samples obtained on admission showed no Y enterocolitica agglutinins Sent home 4 days later Not seen since

Case 15 Woman aged 31 In fourth month of pregnancy In May 1966 she consulted a general practioner because of erythema nodosum She might have had gastro intestinal symptoms ome weeks previously Chest X ray normal appearance Otherwise healthy Culture of faeces gave growth of Y enterocolitica Agglutinin titre against Y enterocolitica 1 160 unchanged titre 6 weeks later The patient then felt well She was later delivered of a healthy child at term

Case 16 Woman aged 26 Since 1963 she had had spells of diarrh ea Treated at hospital (Karlshamn) on two occasions in 1965 and on two in 1966 because of su pected ulcerative colitis Treated with salazosulphapyridine without effect then prednisolone continuously for 7 months Exacerbation of symptoms in Dec 1966 Culture of faeces then gave growth of Y enterocolitica Y enterocolitica agglutinin titre 1 320 after 1 month 1 160 after a further 5 months 1 30 Further treatment and clinical course not known

Case 17 Woman aged 26 For 2 years she had occasionally had pain in the region of the left costal arch for which she sought advice at the outpatient department of her local hospital (Oskarshamn) in Feb 1966 She had had a cold some days previously Because of her hi h E.S.R (62 mm 1 hour) she was admitted for observation Physical examination revealed nothing abnormal Gynaecological examination showed nothing remarkable X ray chest and maxillary sinus normal appearances The agglutinin titre against Y enterocolitica wa incidentally found to be high (1 1280) Culture of faeces because of thi finding gave growth of Y enterocolitica Treated for 1 week with tetracycline Sent home symptomfree Agglutinin titre 3 weeks later 1 320

Case 18 Man aged 25 In Jan 1967 he was admitted to Karlskrona hospital because of gonarthritis Gonococcal complement fixation negative Y ente litica agglutinin titre 1 320 Culture of faeces be ause of the titre gave growth of Y enterocolitica Other ubjective ymptoms not kn wn Treated with demethyl hlor

tetracy line sympt mfree after 3 weeks Agglutinin titre 3 weeks after admission 1 80 2 months after admission < 1 10

Case 19 Woman aged 5 In Feb 1967 she had mild gastro intestinal symptoms with diarrhoea and abdominal pain for 3 days The patient had recently been delivered of a healthy child The symptoms disappeared spontaneously Culture of faeces gave growth of Y *enterocolitica* Y *enterocolitica* agglutinin titre third day less than 1 10 4 weeks later 1 160 Then no persistent symptoms

Case 20 Woman aged 23 In Feb 1967 diarrhoea with body temperature of up to 39 8 C and pain in right iliac fossa After 4 days the fever disappeared but 2 days later the body temperature again rose to about 38 0 C. Then subfebrile for about 2 weeks during which she had moderate diarrhoea Culture of faeces 2 weeks after onset gave growth of Y *enterocolitica* Y *enterocolitica* agglutinin titre was then 1 5120 Treated with sulphametoxipyrimidine After about 2 weeks symptomfree Review 2 months after onset revealed nothing remarkable *High agglutinin titre* against Y *enter colitica* (1 5120) persisted 6 weeks after onset 1 month later 1 1280 7 months later 1 40

Case 21 A woman aged 22 In Feb 1967 diarrhoea fever and colic for a week She afterwards had pain in the elbows knees ankles and hand joints neck and lower back Fourteen days after onset of the initial symptoms erythema nodosum appeared on forearms and lower extremities Admitted to hospital (Malmo) On admission the woman was subfebrile the joints appeared normal The patient was in the se ond to third month of pregnancy Mantoux positive 100 IU antistrepto lysin test negative Chest X ray normal appearance Waaler Rose negative Culture of throat swab negative Culture of faeces gave abundant growth of Y *entero colitica* agglutinin titre on admission 1 1280 Treated for one week with oxitetra cycline and one week later she was symptomfree Sent home after about 3 weeks in hospital Agglutinin titre 8 weeks after onset 1 320 Parturition uncomplicated in Aug 1966 Review 14 months later revealed nothing remarkable

Case 22 A man aged 29 Hitherto healthy Two months previously the patient s wife had been admitted to hospital because of suspected appendicitis Y *entero colitica* had then been isolated from her appendix In Jan 1967 the man had an attack of diffuse abdominal pain accompanied by fever with a max temp of 38 6 C and diarrhoea Mild symptoms of infection of upper airways Two days later ex amined at outpatient department where samples were obtained for culture of the faeces Examination revealed nothing remarkable except meteorism Culture gave growth of Y *enterocolitica* Y *entero olitica* agglutinin titre one month after onset of symptoms 1 1280 9 weeks after onset 1 160 when he felt well

Case 23 Man a d 16 In Oct 1966 symptoms of upper re piratory tract disease fever with temperatures up to 38 5 C and a sore throat. Abdominal discomfort and loose stools once or twice a day He was admitted to hospital (Karlskrona) where he was treated for 10 days with penicillin during which he had chills max temp 38 7 C and extra systole Antistreptolysin test negative Waaler Rose negative E S R. one week after onset of symptom 19 mm/1 hour Incidentally an agglutinin titre of 1 160 against Y *enterocolitica* was found 2 weeks after the onset after a further 2 weeks it was 1 640 and after a further 2 weeks 1 320 Faeces was therefore al o cultured and gave growth of Y *ente ocolitica* Treatment with penicillin was withdrawn and the patient was given demethylchlortetracycline for one week in ste d After 3–4 weeks the patient felt well At review 6 weeks after onset of the disease electrocardiography showed certain abnormalities which were interpreted clinically a myocarditis E.S R *4 mm/1 hour No later data available*

C se 24 Girl aged 15 In May 1966 the girl was seen by a general practitioner becau e of a ore throat with fever and diarrhoea Culture of the faeces gave growth of Y *ente ocol tica* agglutinin titre 1 320 after about 10 days by when patient was symptomfree No other data available

Ca e A boy aged 6 In Sep 1966 onset of fever with a max temp of 39 4 C diarrhoea and abdominal pain The diarrhoea continued for 10 days after which he was admitted to hospital (Karlskrona) Temperature on admission 38 C Chest X ray normal appearance 3–4 days after admission the patient was symptomfree Culture of the faeces gave g owth of Y *enterocolitica* agglutinin titre 3 weeks after n et of symptoms 1 ?0 1 month later 1 160 Patient then treated for one week with phthalylsulphathiazole Review about 2 months after onset revealed nothing remarkable

Case 26 Boy aged 3 In Sep 1966 blood stained diarrhoea and fever The follow

ing day a practitioner was consulted *After collection of faeces for culture chlor* quinald I and phthalylsulphathiazole were given Culture gave growth of Y *entero colitica* Three days later the patient was symptomfree No demonstrable agglutinins 2 weeks after onset Titre 4 weeks after onset 1 10 Review 3 months after onset revealed nothing remarkable

Case 27 A girl aged 2 In April 1966 attack of diarrhoea but no fever Admitted 10 days later to hospital (Karlskrona) After dietary treatment for about one week she was sent home No antibacterial therapy Culture of faeces gave growth of Y *enterocolitica* Not examined serologically Examination one week later *revealed* nothing remarkable

Case 28 A girl aged 2 In Feb 1967 the girl was cared for at hospital (Karlskrona) for 14 days because of acute bronchopneumonia and enterocolitis In March 1967 a new spell of fever with a *max temp of 39 C symptoms of infection of the* respiratory tract sore throat and some days later diarrhoea but no vomiting Re admitted after symptoms for about 5 days Temperature on admission 39 5 C Treated with penicillin for one week Chest X ray normal appearance Culture of faeces gave growth of Y *enterocolitica* No serological analysis Symptomfree after about one week Re examination 3 weeks after onset revealed nothing remarkable

Case 29 A boy aged 2 years In Feb 1967 fever with a max temp of 39 ? C and vomiting The following day he was afebrile but the day after the body temperature again rose this time to 39 5 C and he had severe diarrhoea Admitted to hospital (Karlskrona) where he was given dietary treatment He felt better after a week Culture of the faeces gave growth of Y *enterocolitica* No serological analysis When seen again 14 days later he appeared well

Case 30 Boy aged 11 months In Dec 1966 he was admitted to hospital (Karlskrona) because of diarrhoea No fever *Dietary treatment for one week* cnt home improved Culture of faeces gave growth of Y *enterocolitica* No serological studies He was re admitted 5 days later because of cough and a recurrence of diarrhoea He was still afebrile Treated for 1 week with sulphaisodimidine Re examination one month after *initial onset of the symptoms revealed nothing remarkable*

Case 31 Boy aged three months and a half bottle fed Admitted in Jan 1967 to hospital (Malmo) after 3 days of watery diarrhoea and vomiting No fever Dietary treatment for one week produced no improvement Culture of faeces gave *abundant growth of Y enterocolitica* Treated with demethylchlortetracycline and then diarrhoea decreased and apart from vomiting a few days afterwards he steadily improved with increase in bodyweight One month after admission he was sent home symptomfree Agglutinin titre against Y *enterocolitica* 5 days after admission 1 20 titre after 5 weeks 1 10 Re examination 2 months after onset of the symptoms revealed nothing remarkable

REFERENCES

1 *Carlsson M Ryd H & Sternby N H* A case of human infection with *Pasteurella pseudotuberculosis* Y Acta path et microbiol scandinav 6? 1?8-13? 1964

2 *Frederiksen W* *Personal communications* 1967

3 *Graux C & Wauters G* *Infection familiale a Yersinia enterocolitica* Acta clin belg 21 206-?15 1966

4 *Hugh R & Leifson F* The taxonomi significance of fermentative versus *oxidative metabolism* of carbohydrates by various Gram negative bacteria J Bact 66 ?4-?6 1953

5 *Hassig A Karrer J & Pusterla F* Uber Pseudotuberculose beim Menschen Schweiz med Wschr 79 971-973 1949

6 *Juhlin I & Ericson C* A new medium for the bacteriologic examination of stools (LSU agar) Acta path microbiol scandinav 5? 18?-?00 1961

7 *Ker oncuf A* L érythème noueux à bacille de Malassaz et Vignal These pour le Doctorat en Medicine Faculté de Médicine de Paris 1-118 1967

8 *Lowe G H & Ewans J H* A simple medium for the rapid detection of Salmonella like paracolon organisms *J Clin Path 10* 318-3?1 1957

9 *Mollaret H H* Une nouvelle cause possible d erythème noueux Le bacille de Malassez et Vignal *Pasteurella pseudotuberculosis* Presse méd 70 19?3 196?

10 *Mollaret H H Chevalier J & Deplanche M L* Contribution à l'etude d'un noveau groupe de germes proches du bacille de Malassez et Vignal I Caractères culturaux et biochimiques Ann Inst Pasteur *107* 121-127 1964
11 *Mollaret H H & Destombes P* Les germes X en pathologie humaine Presse méd *72* 2913 2915 1964
12 *Morger R* Zur appendizitischen Form der Pasteurella pseudotuberculosis Infektion beim Kind Praxis *51* 142-144 1962
13 *Nilehn B* Studies on *Yersinia enterocolitica* Characterization of 28 strains from human and animal sources Acta path microbiol scandinav *69* 83 91 1967
14 *Nilehn B* Bacteriological studies on 80 strains of *Yersinia enterocolitica* of human origin Proc 1st Meeting on Pseudotuberculosis Paris 1967 In press
15 *Nilehn B & Sjostrom B* Occurrence of *Yersinia enterocolitica* in patients admitted to hospital because of suspected acute appendicitis The XVth scand Congr Path Microbiol Copenhagen Acta path microbiol scandinav Suppl 187 77-78 1967
16 *Nilehn B & Sjostrom B* Studies on *Yersinia enterocolitica* Occurrence in various groups of acute abdominal disease Acta path microbiol scandinav *71* 612-629 1967
17 *Rappaport F Konforti N & Navon B* A new enrichment medium for certain Salmonellae J clin Path *9* 261-266 1956
18 *Rappaport F & Konforti N* Selective medium for paratyphoid bacteria Appl Microbiol 7 63-66 1959
19 *Schafler S* Influences of some medium conditions on the manifestation of fermentative mutations Z allgem Mikrobiol 2 320-323 1962
20 *Wauters G & Mollaret H H* Un noveau cas d'infection humaine à *Yersinia enterocolitica* Rev belg Path *31* 328-333 1965
21 *Winblad S Nilehn B & Jonsson M* Two further cases bacteriologically verified of human infection with *Pasteurella X* (Syn *Yersinia enterocolitica*) Acta path microbiol scandinav 67 537-541 1966
22 *Winblad S Nilehn B & Sternby N H* *Yersinia enterocolitica (Pasteurella X)* in human enteric infections Brit med J 2 1363-1366 1966
23 *Winblad S* Personal communications 1967

Acta path microbiol scandinav 74 114–126 1968

The Department of Oral Histopathology and the Department of Pathology II
University of Umeå Umeå 6 Sweden

ULTRASTRUCTURAL VASCULAR AND MAST CELL CHANGES IN THE LOCAL *SHWARTZMAN REACTION*

By

GUNNAR T GUSTAFSON

Received 14 iii 68

In a previous light microscopical study (*Gustafson & Cronberg* 1963) endotoxin was found to have no direct effect on mast cells in the peritoneal cavity of rats and hamsters or on mast cells in the hamster cheek pouch As the cheek pouch mucosa injected with the endotoxin nonetheless was found to be prepared for the local Shwartzman reaction it was concluded that endotoxin—mast cell interaction did not mediate the *hemorrhagic necrosis*

However it may be objected that the mast cells may have been altered by the preparatory endotoxin injection although the changes might be so subtle that they could not be observed with the light microscopical technique used *Selye* (1966) commenting upon the results obtained by *Gustafson & Cronberg* speculated that under certain circumstances the degranulation may so rapidly be followed by regranulation that the response can easily be missed

Therefore an ultrastructural study was undertaken to investigate the effect of endotoxin on mast cells of the hamster cheek pouch and *also on the immediate effect of endotoxin on isolated peritoneal mast* cells from rats mice and hamsters In addition the alterations appearing during the development of the local Shwartzman reaction were followed

MATERIALS AND METHODS

Most experiments were performed in adult male and female Syrian hamsters weighing 80–100 g For the *in vitro* experiments peritoneal cells were collected in plastic tubes from hamsters from Sprague Dawley and Wistar rats (200 300 g) and albino mice (20–25 g) The animals were anesthetized with ether and were bled from the carotid arteries Then 7–10 ml of a modified Krebs Ringer solution pH 7 4 without calcium *but containing* 5.5 mM sodiumcitrate (*Chakravarty et al* 1967) or in some experiments Tyrode s solution containing 50 IU ml heparin was injected into the peritoneal cavity The abdomen was cautiously massaged for approximately 90

This investigation was supported by grants from the *Odontological Faculty* University of Umeå



Fig 1

Venule in a 20 hour preparatory lesion in the hamster cheek pouch. The mast cells (arrows) appear quite normal One μ thick CMA section stained with ruthenium red and fast green × 1400

se onds opened and the mixed cell susjension was sucked up and concentrated by centrifugation at 40–60 g for 6 min

Esch ri hia coli and *Salmonella abortus equi* lipopolysaccharides were obtained from Difco Laboratories (Detroit Michigan) In addition endotoxins were prepared from many strains of oral fusobacteria as well as from two strains of oral an aerobic Gram negative cocci as described by *Westphal et al* (1952) or as described by *Taub r & Carson* (1959) The biological activities of these preparations were found to be similar to or slightly weaker than those of the Difco endotoxins when assayed for the capacity to produce local Shwartzman and Thomas reactions in rabbits

In *in vitro* experiments fresh serum from rabbit guinea pig or man was used in a final concentration of 5 per cent as a complement source

Lo al Shwartzman reaction was produced in the hamster cheek pouch by injecting 10 25 μg of the bacterial polysacchari le preparations dissolved in 0 1 ml of physio l gic sal ne 20 hrs later 25 50 μg of the same or other bacterial polysaccharides dissolved in 0 5 ml of physiologic saline were injected intracardially or in some case intraperitoneally

For the *in itro* experiments perit neal cells were collected and pooled from 2 rats 4 hamsters or 8 mice and resuspended in 3 ml of a buffer solution of the ame composition as the isolation solution except that sodium citrate was omitted and replaced by 2 5 mM CaCl and 5 mM glucose and human serum albumin was added to 1 mg/ml (final pH 7) The suspension was incubated at 37 C and then the te t solutions contain ng 0 1–1000 μg endotoxin/ml were added and incubation was c ntinued for 5 15 minutes bef re cooling to 4 C in ice bath



Fig. 2



Fig 3

A hemorrhagic and thrombotic response produced 6 hours after the provocative endotoxin injection The vessel is occluded by disintegrating polymorphonuclear leucocytes and no cell boundaries can be seen The endothelial cell cytoplasm is broken down whereas the nucleus (N) is only slightly affected × 10000

Fig 2

An electr n mi rograph from the same specimen as in Fig 1 Around the vessel one fibroblast (F) and one eosinophilic leucocyte (EO) are seen These cells appear quite normal Other cells one polymorph nuclear leucocyte (PMN) two mono nu lears (MO) and ne mast cell (MA) show small scattered endoplasmic vacuoles × 9000 All electron micrographs were taken from specimens fixed in glutaral lehyde c ntaining 0 001 M p-chloromercuribenzoate GMA se tions were stained with ruthenium red uranyl acetate and lea l

Rabbit peritoneal polymorphonuclear leucocytes were harvested after intraperitoneal injection of 300 ml of 0.1 per cent glycogen concentrated by centrifugation washed in cold 0.34 M sucrose and finally resuspended and homogenized in a Potter homogenizer. When opalescence and viscosity appeared maximal the cell debris was removed by centrifugation at 40 g and the granule fraction finally collected by centrifugation at 17000 g for 20 minutes. For injection into the rabbit skin and the hamster cheek pouch the granule preparation was resuspended in 0.85 per cent NaCl to a volume representing approximately a tenfold concentration of the original peritoneal leucocyte exudate. Although difficult to estimate 1 ml of the granule suspension was considered to represent the granule content from about 10×10^6 leucocytes. A volume of 0.1 ml of the granule preparation was injected into the hamster cheek pouch and 0.5 ml of the same preparation was given in the rabbit skin. Six hrs later the hamsters were injected with 25 μg endotoxin intracardially and the rabbits with 100 μg endotoxin intravenously.

The following procedure was used for isolation of mast cell and leucocyte granules. A fairly pure mast cell fraction (containing 90 ± 5 per cent mast cells) was obtained by isolating mast cells from the peritoneal cell suspension by a method described by *Thon & Uvnäs* (1966) based upon differential centrifugation in 30 and 40 per cent Ficoll, a semisynthetic polysaccharide (Pharmacia AB, Uppsala, Sweden). After ultrasonic treatment to disrupt the mast cells the cell organelles were fractionated as described by *Lagunoff et al* (1964). The granule fraction obtained from in all 60 rats was counted and suspended in 3 ml phosphate buffered saline pH 7.1. After freezing and thawing an amount of 0.1 ml of this suspension containing approximately 10×10 granules was injected into the hamster cheek pouch and 0.5 ml in the skin of rabbits. Six hrs later the hamsters were injected with 25 μg endotoxin intracardially and the rabbits with 100 μg endotoxin intravenously.

The fixation, staining and electron microscopic techniques were the same as described previously (*Gustafson & Pihl* 1967a, b, c). In most series cell and tissue specimens were fixed for 3 hrs in 4 per cent methanol free formaldehyde or 2.5 per cent glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, with 0.001 M p-chloromercuribenzoate (PCMB) (Sigma Co., St. Louis, U.S.A.).

Other specimens were fixed in a solution of the same composition but containing in addition 0.001 M ruthenium red. Dehydration and embedding were done in the water soluble glycolmethacrylate (GMA) obtained from Rohm and Haas, Darmstadt, Germany (*Rosenberg et al* 1960, *Leduc et al* 1963, *Leduc & Bernhard* 1967).

One μ thick and ultrathin sections were stained with 0.001 M ruthenium red in 0.1 M tris maleate buffer, pH 7.4, and in some cases also in phosphate buffer pH 7.4 for 15 minutes. One μ thick sections were also stained with a 0.5 per cent solution of toluidine blue and in some series with a 0.1 per cent solution of berberin sulphate and examined in a fluorescence microscope. Ultrathin sections were cut on LKB Ultramicrotomes and collected on formvar-coated grids.

Other specimens were fixed in a solution containing the same components as that described but containing in addition 0.001 M ruthenium red. Sections from these specimens were washed in a tris maleate buffer, dehydrated in alcohol and embedded in Epon 812 (*Luft* 1961). Ultrathin sections from this material were collected on unsupported copper grids and studied without further treatment or after staining with lead citrate and uranyl acetate. Cell suspensions were treated in a similar way for light and electron microscopy as described earlier (*Gustafson & Pihl* 1967a, b, c, *Chakravarty et al* 1967). The ultrathin sections were examined in a Siemens Elmiskop I a at original magnifications varying from 5000 × to 40000 ×.

RESULTS

Neither light microscopy nor electron microscopy revealed any changes in the structure of mast cells during the first hours after application of the endotoxin preparations. After twenty hours immediately before the eliciting injection most mast cells were unaffected but some appeared somewhat shrunken in the light microscope (Fig. 1). In the electron microscope mast cells as well as other types of cells in the environment had acquired many small vacuoles (Fig. 2). However

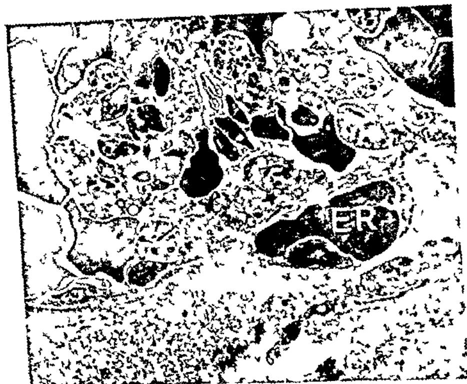


Fig 4

A venule occluded by erythrocytes and thrombocytes 8 hours after the provocative endotoxin injection One erythrocyte (ER) is situated intramurally and has forced the endothelial cell nucleus aside × 8100

most mast cell granules appeared quite normal and there was no indication that a mast cell degranulation had taken place or was going on The mast cells did not look more damaged than did other cells

Immediately after the intravenous eliciting dose of endotoxin the venules were found to be packed with neutrophilic leucocytes (Fig 3) the cell boundaries of which were obviously broken up The leucocyte granules seemed to be released directly into the surrounding connective tissues as the vessel walls were generally disintegrating In other parts of the damaged area the vessels were engorged with thrombocytes and erythrocytes (Fig 4) The vessel walls contained intramural erythrocytes and thrombocytes obviously situated between endothelial cells and pericytes In the extravascular space disintegrating collagen fibres and vacuole filled histiocytes could be seen That not only the cytoplasmic structures were damaged is shown in Fig 5 in which the nucleus of a mononuclear cell is disintegrating The mast cells (Fig 6) generally appeared to be less damaged than most other types of cells but more altered than the eosinophilic leucocytes The vacuolization of mast cells was slight compared to that of histiocytes in the neighbour

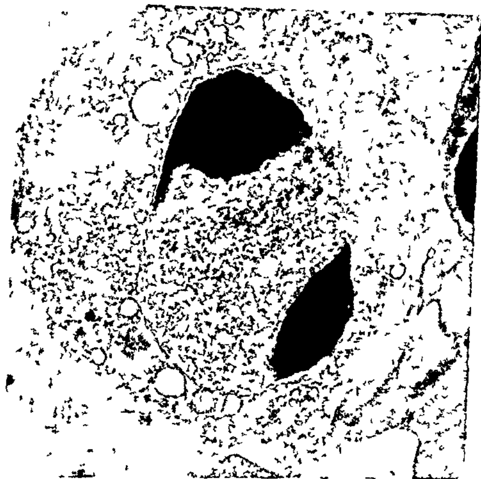


Fig 5

A mononuclear cell from a hemorrhagic reaction 8 hours after the provocative endotoxin injection. The figure shows that mononuclear cells are also subject to autolytic changes × 16000

hood. No signs of degranulation typical of histamine release (*Bloom & Haegermark* 1965, *Chakravarty et al* 1967) were observed among the mast cells within the necrotic area studied.

When isolated peritoneal mast cells were exposed to different concentrations of many endotoxin preparations (0.1–1000 µg/ml) these cells did not appear different from those exposed to the buffer solutions. Addition of serum from guinea pig, rabbit or man did not change the result. Corresponding results were obtained with peritoneal cells from rats and mice.

When disrupted granules of polymorphonuclear leucocytes were injected into rabbit skin and into hamster cheek pouch mucosa, a hemorrhagic reaction was obtained in most cases (8 out of 12 hamsters and

6 out of 10 rabbits). When the same procedure was performed with mast cell granules in 10 hamsters no such reactions were seen.

In 6 rabbits which had been "prepared" with leucocyte granules on one side of the abdomen and with mast cell granules on the other 4 rabbits presented positive reactions on the "leucocyte" side but were negative on the "mast cell" side after the eliciting endotoxin injection.

DISCUSSION

In the preceding light microscopical study of the effects of endotoxin on mast cells it was concluded that endotoxin did not seem to have any direct immediate effects (*Gustafson & Cronberg* 1963). The same conclusion seems to be justified by the results obtained in the electron microscopical study presented here. There was no indication that mast cells had reacted in an anaphylaxis-like way with degranulation and release of granules to the surrounding tissues. This finding was further substantiated by the results obtained in the studies of the effect of endotoxin on peritoneal mast cells *in vitro* irrespective of whether serum from rabbit, guinea pig or man was added as a complement source or not. Thus the vasoactive amine release reported to follow endotoxin injection (reviewed by *Hinshaw* 1964) does not seem to originate from anaphylactic histamine or serotonin release from mast cells. These results are similar to but not identical with those obtained by *Taichman et al.* (1966). These authors reported the preparative endotoxin injection to have no effect on mast cells after 20–24 hours. However, that finding was not illustrated by electron micrographs. In the present study the mast cells as well as other cells obviously had been altered although there was no degranulation and the alterations were too subtle to be clearly observed in the light microscope.

The dramatic effect of the eliciting intravenous endotoxin injection did not seem to affect the mast cells to any marked degree. The central role played by leucocytes of myeloic origin has been repeatedly demonstrated (*Becker* 1948, *Stetson & Good* 1951) although the exact role played by these leucocytes is obscure. One possibility is that the granule (lysosomal) enzymes are released and disrupt the vessel walls (*Thomas* 1964, *Halpern* 1964, *Burke et al.* 1964). Cationic proteins and other constituents of the polymorphonuclear granule have also been shown to have permeability increasing effects on vessels (*Janoff & Zweifach* 1964, *Zeya & Spitznagel* 1966). The isolated leucocyte basic material causes a delayed increase in vascular permeability and hemorrhagic Arthus-like reactions (*Golub & Spitznagel* 1964, *Golub & Spitznagel* 1966) and was found to contain an agent capable of rupturing and degranulating mast cells (*Janoff et al.* 1965). The mastolytic agent labelled with fluorescein and injected subcutaneously into mice was shown to be bound to mast cell granules liberated as a consequence of the mast cell rupturing basic material (*Clark & Higginbotham* 1966).

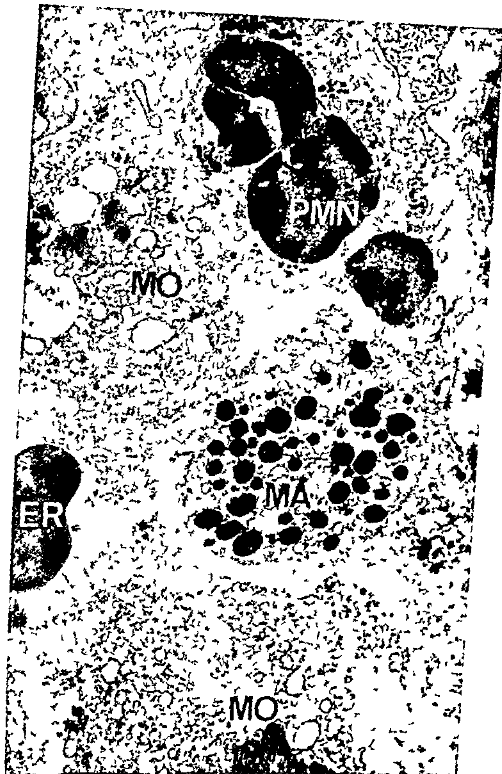


Fig 6

It has also been questioned if mast cell disruption might be able to more directly mediate immune vasculitis (*Humphrey* 1966) as rabbits depleted of polymorphonuclear leucocytes have been shown to have a residual capacity to produce this type of immune injury (*Cochrane & Ward* 1966)

In the present ultrastructural study there was no indication that rupturing of mast cells may represent a common outcome in the final necrotic processes produced by endotoxins It should be emphasized however that mast cells in the necrotic area were rather few In addition some mast cells were so disintegrated that they could be recognized as mast cells with certainty in the electron microscope only thanks to the specific staining of the granule glycosaminoglycans with ruthenium red (*Gustafson & Pihl* 1967 a c)

The finding that isolated mast cell granules in contrast to those of the leucocytes did not produce any signs of hemorrhagic necrosis in hamsters and rabbits injected with endotoxin suggests that mast cell degranulation does not mediate the local Shwartzman reaction However the amount of mast cell granules might have been to small and the loss of enzyme and other granule constituents great Therefore the negative results obtained with mast cell granules might be caused by experimental faults and the possibility cannot be completely excluded that granules released *in vivo* might prepare for hemorrhagic vasculitis

It has been speculated that the reactivity against bacterial endotoxins might depend upon the existence of natural antibodies to Gram negative bacteria (*Landy & Weidanz* 1964) These antibodies have generally been considered to be γM antibodies (*Landy & Weidanz* 1964 *Michael & Rosen* 1964) but γA and γG antibodies to Gram negative bacteria have also been found (*Cohen & Norins* 1966 *Altemeier et al* 1966) Recently *Gupta & Reed* (1967) using a modified method developed by *Farr* (1958) to measure the amount of endotoxin antibody complexes formed in half saturated ammonium sulfate also found not only 19S but also 7S antibodies against Salmonella enteritidis endotoxin in normal human and rabbit sera That antibodies related to γG globulins may have been missed and underestimated in relation to γM antibodies in earlier studies may be due to the fact that γM antibodies are many times more effective in producing bacterial agglutination and more potent as sensitizers of bacteria in complement dependent cytolysis than are γG globulin antibodies (*Robbins et al* 1965) The results obtained in the present investigation do not indicate that antibodies

Fig 6

Local Shwartzman reaction 6 hours after elicitation Vacuolized and disintegrating mononuclear cells (MO) and one polymorphonuclear leucocyte (PMN) are seen around a slightly affected mast cell (MA) An extravasated erythrocyte (ER) is seen × 10800



Fig 6

Farr R S A quantitative immunochemical measure of the primary interaction between I * BSA and antibody J Infect Dis *103* 239 262 1958

Golub F S & *Spitznagel J K* Dermal lesions induced by homologous PMN lysosomes Fed Proc *23* 509 1964

Golub E S & *Spitznagel J K* The role of lysosomes in hypersensitivity reactions Tissue damage by polymorphonuclear neutrophil lysosomes J Immunol *95* 1060 1066 1966

Gupta J D & *Reed C F* The direct reaction between Salmonella enteritidis endotoxin and antibody Measurement of 7S and 19S antibody in normal tolerant and immune sera J Immunol *98* 1093-1103 1967

Gustafson G T & *Cronberg S* Effect of endotoxin on mast cells and the extension of the local Shwartzman reaction to the hamster Acta path microbiol scandinav *59* 21-31 1963

Gustafson G T & *Pihl E* Histochemical application of ruthenium red in the study of mast cell ultrastructure Acta path microbiol scandinav *69* 393-403 1967a

Gustafson G T & *Pihl E* The effect of heavy metal and aldehyde fixatives on normal and injured mast cells Abstract J Ultrastruct Res *20* 300 1967b

Gustafson G T & *Pihl E* Staining of mast cell acid glycosaminoglycans in ultrathin sections by ruthenium red Nature *216* 697-698 1967

Halpern B N Inhibition of the local hemorrhagic Shwartzman reaction by a polypeptide possessing potent antiprotease activity Proc Soc exp Biol *115* 273-276 1964

Hinshaw L B The release of vasoactive agents by endotoxin In Bacterial Endotoxins Ed Landy M & Braun W Rutgers University Press New Brunswick N J 1964

Humphrey J H In Immunopathology IVth International Symposium Ed Grabar P & Miescher P A p 458 Schwabe & Co Basel 1966

Janoff A & *Zweifach B W* Production of inflammatory changes in the microcirculation by cationic proteins extracted from lysosomes J exp Med *120* 747-764 1964

Janoff A *Schaefer S* *Scherer J* & *Bean M A* Mediators of inflammation in leukocyte lysosomes II Mechanism of action of lysosomal cationic protein upon vascular permeability in the rat J exp Med *122* 841 851 1965

Landy M & *Weidanz W P* Natural antibodies against gram negative bacteria In Bacterial Endotoxins Ed Landy M & Braun W Rutgers University Press New Brunswick N J p 275-290 1964

Lagunoff D *Phillips M D* *Iseri O Z* & *Benditt E P* Isolation and preliminary characterization of rat mast cell granules Lab Invest *13* 1331-1344 1964

Leduc E H & *Bernhard W* Recent modifications of the glycol methacrylate embedding procedure J Ultrastruct Res *19* 196-199 1967

Leduc E H *Marinozzi V* & *Bernhard W* The use of water soluble glycol methacrylate in ultrastructural cytochemistry J Roy Micros Soc *81* 119-130 1963

Luft J H Improvements in epoxy resin embedding methods J Biophys Biochem Cytol *9* 409-414 1961

Michael J G & *Rosen F S* Physicochemical characterization of naturally occuring antibodies to Gram negative bacteria In Bacterial Endotoxins Ed Landy M & Braun W Rutgers University Press New Brunswick N J p 306-310 1964

Mota I Mechanism of anaphylaxis I Production and biological properties of mast cell sensitizing antibody Immunology 7 681-699 1964

Robbins J B *Kenny K* & *Suter E* The isolation and biological activities of rabbit γM and γG anti Salmonella typhimurium antibodies J exp Med *122* 385-402 1965

Rosenberg M *Bartl P* & *Lesko J* Water soluble methacrylate as an embedding medium for the preparation of ultrathin sections J Ultrastruct Res 4 298-303 1960

Selye H The Thrombohemorrhagic Phenomena Charles C Thomas Publisher Springfield p 262 263 1966

Stetson C A & *Good R A* Studies on mechanism of Shwartzman phenomenon evidence for participation of polymorphonuclear leucocytes in the phenomenon J exp Med *93* 49 64 1951

Tada T & Ishizaka K Arthus type inflammation with rabbit γM antibody J Immunol *96* 112–118 1965
Taichman N S Uriuhara T & Movat H Z Ultrastructural alterations in the local Shwartzman reaction Lab Invest *14* 2160–2176 1966
Tauber H & Garson W Isolation of lipopolysaccharide endotoxin J Biol Chem p 1391–1393 1959
Thomas L Possible role of leucocyte granules in the Shwartzman and Arthus reactions Proc Soc exp Biol *115* 235–240 1964
Thon I I & Uvnäs B Mode of storage of histamine in mast cells Acta Physiol Scand *67* 455–470 1966
Westphal O Lüderitz O & Bister F Über die Extraktion von Bakterien mit Phenol/Wasser Z. Naturforsch *7b* 148–155 1952
Zeya H I & Spitznagel J K Cationic proteins of polymorphonuclear leukocyte lysosomes I Resolution of antibacterial and enzymatic activities J Bact *91* 750–754 1966

Acta path microbiol scandinav 74 127-138 1968

The Department of Oral Histopathology and the Department of Pathology II University of Umeå Umeå 6 and the Wallenberg Laboratory University of Uppsala Uppsala Sweden

HEMORRHAGIC REACTIONS IN THE HAMSTER PRODUCED BY INTERACTION OF PROTEIN A FROM *STAPHYLOCOCCUS AUREUS* HUMAN γ GLOBULIN AND ENDOTOXIN

By

GUNNAR T GUSTAFSON

Received 14 iii 68

In a previous communication (*Gustafson et al* 1967) it was shown that *hemorrhagic* Arthus like reactions were produced in rabbits by the interaction of human γG globulin and a cell wall protein (protein A) from *Staphylococcus aureus* The skin lesions were produced either by intradermal injection of preformed protein A—γ globulin complexes or by intradermal injection of protein A into rabbits that had previously received human γ globulin intravenously The skin reactions were considered not to be caused by an antigen antibody reaction but rather to a pseudoimmune reaction between protein A and human γ globulin

In the course of trying to repeat the experiments in other species it was found that the results could not be reproduced in hamsters If human γ globulin and protein A were given to hamsters in equivalent or higher doses than those given to rabbits a slight erythema developed but no hemorrhagic reactions ensued

In a preceding report (*Gustafson* 1968) the possibility was considered that the preparative effect of endotoxin for the local Shwartzman reaction was due to an interaction between *precipitating* antibodies to Gram negative bacteria and the endotoxin preparations As it is also wellknown that intracutaneous injection of the homologous antigen in sensitized animals prepares for the local Shwartzman reaction (reviewed by *Thomas* 1959) the slight inflammatory process produced by human γ globulin and protein A in the hamster cheek pouch *might possibly* be converted into a hemorrhagic vasculitis by an intravenous endotoxin injection The present study reports the results

This investigation was supported by grants from the *Odontological Faculty* University of Umeå *the Swedish Cancer Society* (Project No 50-B67 02X) and the *Swedish Medical Research Council* (Project No K67-13X-690-03A)

of such experiments The development of the reactions was followed by means of vital and conventional light microscopy as well as by electron microscopy

In order to try to elucidate the role played by γ globulin in the hemorrhagic reaction the interaction between aggregated γ globulin and endotoxin was also studied

MATERIALS AND METHODS

Most experiments were performed in male and female Syrian hamsters weighing 80–100 g For *in vitro* experiments cells were also isolated from Sprague Dawley rats (200–300 g) and albino mice (20–25 g) mainly as previously described (*Chakravarty et al* 1967) For some local Shwartzman reaction experiments rabbits (1 4–1 6 kg) were used Each experiment was performed in series consisting of 6 24 animals and at least 6 controls

The staphylococcal protein (protein A) was prepared as described by *Forsgren & Sjoquist* (1966)

The direct effect of protein A was studied after injection of the protein in amounts from 0 1 μg to 0 4 mg in 0 1 ml of physiologic saline in the hamster cheek pouch mucosa with or without preceding blueing of the animal with 1 mg Evans blue/100 g body weight given intracardially *The effect of the local application of* different concentrations of protein A onto the surface of a microwound in the hamster cheek pouch was also studied and registered in a vital microscope (*Lindhe & Brånemark* 1967) with or without a preceding injection of 15–25 mg of human γG globulin obtained from Kabi AB Stockholm Sweden

In one series of experiment 0 05 mg 0 1 mg 0 2 mg or 0 4 mg of protein A in 0 1 ml buffered saline was injected into the cheek pouch mucosa of 36 hamsters of which 18 had received human γ globulin intravenously or intracardially 5 minutes earlier

After 20–24 hours 12 of the animals given γ globulin and 12 not given γ globulin were in addition injected intravenously or intraperitoneally with 25–50 μg endotoxin from *E coli* *S abortus equi* or *Fusobacterium* species The *in vitro* experimental procedures were the same as previously described (*Gustafson* 1968)

The peritoneal cell suspensions were exposed to different concentrations of protein A (0 01 μg 0 1 μg 1 μg 10 μg 100 μg and 1000 μg/ml) with or without addition of serum from rabbit guinea pig or man mainly as described earlier (*Gustafson* 1968)

In some experiments it was tried to passively sensitize mast cells with heterologous serum and human γ globulin The cells isolated after intraperitoneal injection of a heparin Tyrode solution were centrifuged and resuspended in isotonic saline containing serum or γ globulin and incubated for 1–15 hrs at 4 C 18 C or 38 C as described by *Garcia Arocha* (1966)

Fig 1

Reactions produced by protein A in hamsters not injected with human γ globulin a) Five vessels from hamster cheek pouch are very little altered The perivascular *cells are almost normal* in number and appearance One μ thick CMA section stained with ruthenium red and fast green × 1000 b) This electron micrograph shows a *mast cell* from the same area as in Fig 1a There is no degranulation c) A detail from the mast cell shown in Fig 1b The perigranular as well as *the cellular mem*branes are normal d) A mast cell and a mononuclear cell after an eliciting intracardial endotoxin injection The mononuclear cell has many cytoplasmic vacuoles The specimens in this and following figures were fixed in glutaraldehyde contain*ing* 0 001 M p chloromercuribenzoate embedded in CMA and ultrathin sections stained with ruthenium red uranyl acetate *and lead*

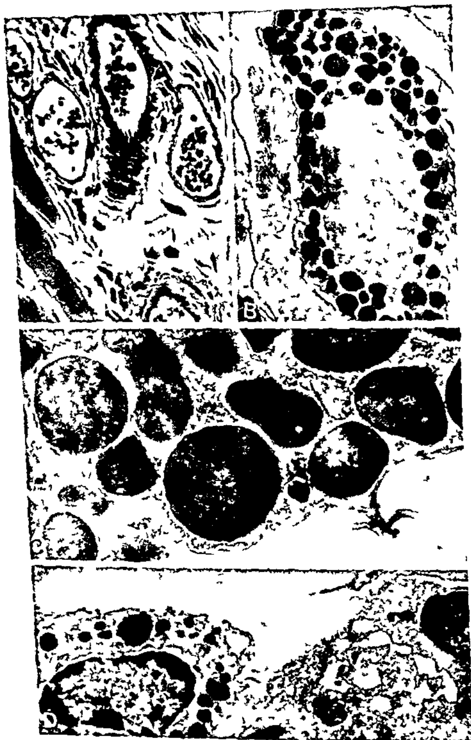


Fig 1



Fig 2

The inflammatory response to protein A 20 hours earlier injected in the cheek pouch of a hamster given human γ globulin The electron micrograph shows that an exudate has developed in the vessel wall The extracellular structure appears to be somewhat disorganized The mast cell (MA) cytoplasm seems to be broken up and the central part of the granules as well as nucleus is disintegrated

Aggregated human γ globulin (Kabi) was obtained by coupling with bis diazotized benzidine (*Ishizaka et al* 1961) In a first series of experiment different amounts of aggregated γ globulin in 0 3 ml buffered saline were injected into the shaved flanks of rabbits After 3–4 hrs the inflammatory reaction generally appeared maximal An amount of 1 2 mg aggregated γ globulin was found to produce besides edema and erythema confluent petechiae in most animals In a second series 18 out of 24 rabbits that had been intracutaneously injected with 1 2 mg of aggregated γ globulin received in addition after different intervals (2 hrs 12 hrs 18 hrs and 24 hr) an intravenous injection of 250 μg of *Escherichia coli* or *Salmonella abortus equi* endotoxin (Difco Laboratory Detroit Michigan)

The fixation staining and electronmicroscopical techniques were the same as previously described (*Gustafson & Pihl* 1967 a b c)

RESULTS

In hamsters not previously injected with human γ globulin the application of protein A solutions to the microwound of the cheek pouch in a concentration of 1 μg–500 μg had no effect on the vessels or microcirculation Nor did injection of 0 1 100 μg of protein A into the skin



Fig 3

Hemorrhagic reactions produced by 0 2 mg protein A in the exposed cheek pouches of a hamster previously given 20 mg γ globulin and in addition 25 μg *Escherichia coli* endotoxin intracardially 20 hours later

and cheek pouch result in any signs of increased capillary permeability in 12 blued hamsters Neither in thick nor in ultrathin sections studied could any definite changes be observed in endothelium mast cells or other cells in the injected area after 20 hours (Figs 1 a b c)

In 12 hamsters injection of endotoxin intracardially 20 hours after the protein A injection did not produce any gross reactions at the prepared site Electron microscopy however revealed that the endotoxin injection had induced vacuolization of many mononuclear cells but mast cells appeared comparably less damaged (Fig 1 d)

The effect of protein A was also studied in the vital microscope in 24 hamsters injected 5–10 minutes earlier with 20 mg human γ globulin The local application of 0 1 ml of a solution containing 10 μg protein A to the cheek pouch wound caused the corpuscular flow to slow down after 8–10 minutes and during the following 20–30 minutes arteriolar constriction and venule dilatation was alternatively intensified and reduced Momentarily the white cell flow predominated and for a few seconds the flow of blood ceased completely This seemed to be a consequence of the increased volume of the endothelial walls and perhaps was also due to the reduced plasticity of the blood cells

After 2–3 hours the microcirculation appeared quite normal In the light microscope the vessels and mast cells looked normal in number

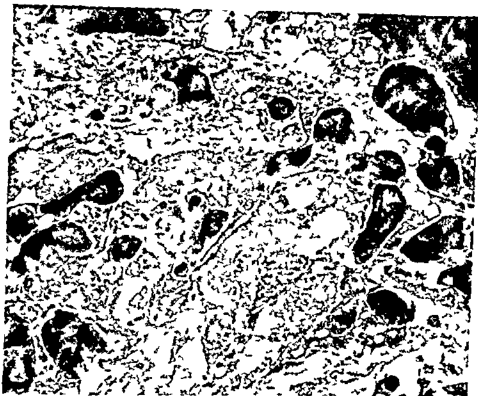


Fig 4

From a hemorrhagic reaction produced in the same way as described in Fig 3 The vessel is occluded by almost totally disintegrated polymorphonuclear leucocytes The vessel walls can no longer be recognized

and appearance After 20 hours however the mast cells generally were slightly shrunken and the endothelial cells somewhat swollen

In the electron microscope more marked changes were observed at this time (Fig 2) Between the endothelial cells and pericytes a space had developed in most vessels that was filled with an unidentified material The endothelial cells pericytes and histiocytes contained many small vacuoles The mast cells also showed cytoplasmic vacuoles their granules often looked more or less emptied and their cell membranes were usually completely destroyed Despite the striking ultrastructural changes after the preparative injection with protein A the gross reactions were only slight and restricted to faint redness and edema of the prepared area of the cheek pouch

---

Fig 5

From a hemorrhagic reaction produced in the same way as described in Fig 3 a) A venule is occluded by erythrocytes and thrombocytes Two erythrocytes (ER) are seen in the extravascular space b) The vessel wall (VW) has broken up and the opening is filled with disintegrating thrombocytes and fibrin



Fig 5

Other animals treated in the same way, i.e. 20 hours earlier given human γ globulin intravenously (20 mg) and protein A (0.2 mg or more) in the cheek pouch, was injected parenterally with endotoxin (25–50 μg). Within 4–7 hours after the injection an intense submucosal *hemorrhage developed (Fig. 3). In the light and electron microscope* the vessels were found to be engorged with disintegrating polymorpho nuclear cells (Fig. 4), erythrocytes and thrombocytes (Fig. 5a). The latter were highly vacuolized and these cells and fibrin often plugged splits in the vessel walls (Fig. 5b). The few mast cells which could now be observed were found to present the same type of injury as after the preparative injection but often they were so damaged that the nucleus as well as the cytoplasm was *completely disintegrated*.

In 18 rabbits injected with aggregated human γ globulin the intravenous injection of 250 μg endotoxin after 2, 12, 18 or 24 hours gave no increase in the area of the hemorrhagic reaction when compared with the reactions obtained in 6 rabbits not injected with endotoxin. However, *rabbits which had received endotoxin after 18 hours seemed to present reactions of somewhat higher intensity than the control rabbits*.

As protein A had markedly affected the mast cells of hamsters in the *in vivo* reactions the effect of protein A on peritoneal cells was studied *in vitro*. Mast cells from hamsters, rats and mice however were not *found to be altered after exposure to protein A in concentrations from* 0.01–1000 μg/ml. The addition of serum from rabbit, guinea pig or man did not change the result. As protein A has been found to precipitate human and guinea pig γ globulins in agar gel in very low concentrations, an attempt was also made to passively sensitize mast cells of hamster, rat and mouse *with human and guinea pig γ globulins*. These experiments all gave negative results. Mast cells incubated with human or guinea pig γ globulin and then exposed to protein A solutions appeared not different from the controls either in the light or in the electron microscope.

DISCUSSION

In rabbits injected with human γ globulin, protein A was found to produce an Arthus like hemorrhagic necrosis when injected into the skin (*Gustafson et al.* 1967). Although, as shown in the present communication, protein A injected into the cheek pouch of hamsters previously injected with human γ globulin produced an inflammatory process, this reaction did not terminate in hemorrhagic necrosis. The cause of the different results obtained in these two species is unknown.

As mild delayed hypersensitivity reactions can be converted into hemorrhagic processes by endotoxin injection (*Stetson* 1959) it may be questioned whether the capacity of protein A to prepare the cheek pouch for hemorrhagic necrosis elicited by endotoxin injection might have been due to delayed hypersensitivity of the hamster to protein A. However, this does not seem probable as no direct gross reactions

were produced in skin or mucosa and as no necrotic reactions developed after intravenous endotoxin injection in animals not previously given human γ globulin

The effect of protein A on mast cells *in vivo* was most probably a secondary effect of the protein A γ globulin complex as protein A could not be shown to produce any alterations in the peritoneal mast cells *in vitro* and as preformed antigen antibody complexes have no histamine releasing or degranulating effect on mast cells (*Mota* 1963 *Keller* 1966) Another possibility is that the protein A preparations were contaminated with α hemolysin which has been claimed to vacuolize rabbit mast cells (*Orfanos* 1966) However the protein A preparations were not found to contain α hemolysin when assayed for this substance (*Gustafson et al* 1968) α hemolysin would also be expected to seriously affect mast cells from hamster rat and mouse *in vitro*

Two possibilities seem to exist for bacterial or other antigens to produce necrotizing processes The first is that the level of natural or acquired antibodies may be high enough to give rise to hemorrhagic Arthus like reactions directly on contact with the antigen in the vessel wall Or if the circulating antibodies are too few to produce hemorrhagic necrosis when meeting the antigen in the vessels a Gram negative bacterial infection may by means of endotoxins released so influence the inflammatory process that hemorrhagic necrosis develops

The result that human γ globulin aggregated in the vessel wall by means of protein A without the involvement of the antigen combining site (*Sjoquist et al* 1967) prepared the hamster mucosa for the local Shwartzman reaction indicates that γ globulin complexes *per se* may be responsible not only for the hemorrhagic vasculitis in Arthus reactions (*Ishizaka* 1963) but also for the preparative effect of the homologous antigen in previously immunized animals in the local Shwartzman reaction (*Thomas* 1959) According to this concept preformed antigen antibody complexes or human γ globulin aggregated by bis diazotized benzidine might be expected to prepare for the local Shwartzman reaction However the inflammatory processes produced by such complexes were not found to be markedly intensified by an intravenous endotoxin injection One explanation may be that the injected complexes were not localized to the vessel walls to the same extent as the complexes formed *in vivo* Thus the vessels were not as susceptible to the thrombosis induced by the eliciting endotoxin injection

By what mechanism does the eliciting endotoxin injection intensify the inflammatory process started 20 hours earlier by the protein A γ globulin interaction? *Michael* (1966) has recently shown that antibodies against bacterial antigens are released from the reticuloendothelial system (R E S) after endotoxin injection In the local Shwartzman reaction released antibodies against O antigen of Gram negative bacteria after the eliciting endotoxin injection might react with endotoxin molecules persisting at the prepared site and thereby convert the

quiet inflammatory process into a hemorrhagic vasculitis If human γ globulin molecules injected into hamsters are similarly concentrated to R Γ S and released by endotoxin injection is unknown It may be speculated that if human γ globulin molecules are released after parental endotoxin injection and react with protein A molecules persisting at the prepared site these might lead to iteration of the initial events produced at the preparation Actually in the vital microscope intravenous endotoxin injection was found to give rise to exactly the same vascular reactions in the prepared area as those which followed the protein A application into the microwounds in hamsters injected with human γ globulin (unpublished observations) However other biological effects of endotoxin such as blocking of R Γ S interferance with polymorphonuclear leucocyte function and potentiation of the effect of catecholamines (*Thomas* 1959) may also be determinant mechanisms *in the development of the reactions seen in the vital microscope as well* as to the final hemorrhagic vasculitis

An accelerated inflammatory response shall not always be regarded as an increased defense mechanism against invading bacteria This fact has been illustrated by experiments described by *Johnson et al* (1961) These authors showed that repeated infection with staphylococci led to hypersensitivity which allowed the establishment of infection by much lower numbers of the microorganisms than was required in non sensitized animals Thus as hypersensitivity states lead to increased infectivity for the same as well as other unrelated infective agents and to intensified inflammation and necrosis a vicious circle is established which may be of importance in the development of infectious disease

Synergistic mechanisms seems to be especially important in Gram negative oral infections such as Vincent s and human bite infections This seems valid also for staphylococcal infections In progressive synergistic bacterial gangrene of the abdominal wall after surgical treatment and in impetigo *Staphylococcus aureus* has been considered to play a predominant role in the mixed infection (*Arndt* 1965) The enhancement of Gram negative infection by *Staphylococcus aureus* has also been demonstrated in experimental studies (*Arndt & Ritts* 1961) Besides the destructive hypersensitivity phenomena produced by endotoxin and *Staphylococcus aureus* as a result of pseudo immune reactions with protein A or true immune reactions involving techoic acids (*Martin et al* 1967) there are of course many other mechanisms which may contribute to the synergism between Gram negative bacteria and *Staphylococcus aureus* such as local consumption of antibody and of complement factors temporary blocking of the reticuloendothelial system by endotoxin (*Thomas* 1957) and increased vascular reactivity to vasoactive amines caused by endotoxins (*Hinshaw* 1964)

SUMMARY

The effect of a cell wall protein (protein A) of *Staphylococcus aureus* injected into the cheek pouch of the hamster was studied by vital and conventional light microscopy and by electron microscopy Protein A by itself had no effect In animals previously given human γ globulin parenterally protein A elicited an inflammatory reaction characterized by sludged blood circulation and temporary thrombosis which started within 10 minutes and subsided after 2–3 hours Examinations 20–24 hours after the preparative injection revealed but subcellular changes especially vacuolization of mononuclear cells Parenteral injection of endotoxin at this time elicited a necrotizing hemorrhagic vasculitis All three steps were necessary to obtain the necrotizing hemorrhagic vasculitis the parenteral injection of human γ globulin the local injection of protein A and the parenteral endotoxin injection

Synergistic infections with *Staphylococcus aureus* and Gram negative bacteria in man are discussed in view of these findings

REFERENCES

Arndt W F Synergistic infections In Skin bacteria and their role in infection Ed Maibach H J & Hildick Smith G McGraw Hill Book Company New York 1965

Arndt W F & Ritts R E Synergism between staphylococci and proteus in mixed infection Proc So exp Biol *108* 166–169 1961

Chakravarty N Gustafson G T & Pihl E Ultrastructural changes in rat mast cells during anaphylactic histamine release Acta path microbiol scandinav *71* 233–244 1967

Forsgren A & Sjoquist J Protein A from S aureus I Pseudo immune reaction with human γ globulin J Immunol *97* 8??–8?7 1966

Garcia Arocha H Histamine release from isolated passively sensitized cells Lecture at the international pharmacological meeting in Sao Paulo 1966

Gustafson G T Ultrastructural vas ular and mast cell changes in the local Shwartzman reaction Acta path microbiol scandinav *74* 114–1?6 1968

Gustafson G T & Pihl E Histochemical application of ruthenium red in the study of mast cell ultrastructure Acta path microbiol scandinav *69* 393–403 1967a

Gustafson G T & Pihl E The effect of heavy metal and aldehyde fixatives on normal and injured mast cells Abstract J Ultrastruct Res *20* 300 1967b

Gustafson G T & Pihl E Staining of mast cell acid glycosaminoglycans in ultra thin sections by ruthenium red Nature *216* 697–698 1967c

Gustafson G T Sjoquist J & Stålenheim G Protein A from Staphylococcus aureus II Arthus like reaction produc d in rabbits by interaction of protein A and human γ globulin J Immunol *98* 11?8–1181 1967

Gustafson G T Stålenheim G Forsgren A & Sjoquist J "Protein A from S aureus VI Production of anaphylaxis like cutaneous and systemic reactions in non immunized guinea pigs In press J Immunol *100* 1968

Hinshaw L B The release of vasoactive agents by endotoxin In Bacterial endotoxins Ed Landy M & Braun W p 118–12? 1964

Ishizaka K Gamma globulin and molecular mechanisms in hypersensitivity reactions Progr Allerg 7 3 –106 1963

Ishizaka K Ishizaka T & Sugahara T Bi logical activity of aggregated γ globulin III I roduction of Arthus like reactions J Immunol 86 2?0–??7 1961

Johns n J E Cluff L F & Goshi K Studies on the pathogenesis of staphylococcal infection I The effect f repeated skin infections J exp Med *113* 23?–?18 1961

Keller R Tissue mast cells in immune reactions Monographs in Allergy Karger Basel 1966

Lindhe J & Branemark P I Changes in microcirculation after local application of sex hormones J periodont Res 2 185–193 1967

Martin R R Crowder J G & White A Human reactions to staphylococcal antigens A possible role of leukocyte lysosomal enzymes J Immunol 99 269–275 1967

Michael J G The release of specific bactericidal antibodies by endotoxin J exp Med 123 205–212 1966

Mota I The behavior of mast cells in anaphylaxis In International review of cytology Ed Bourne G H & Danielli J F Academic Press New York and London 15 363–395 1963

Orfanos C Mastzelle und Mastzelldegranulation Klin Wschr 44 1177–1182 1966

Sjoquist J Forsgren A Gustafson G T & Stålenheim G Interaction between gamma globulins and protein A from Staphylococcus aureus In Gamma globulins Nobel Symposium 3 Ed Killander J p 341–348 1967

Stetson C A Endotoxins and bacterial allergy In cellular and humoral aspects of the hypersensitive states Ed. Lawrence H S Hoeber New York 1959

Thomas L The role of the reticulo endothelial system in the reaction to endotoxins In Physiopathology of the reticulo endothelial system Ed Halpern B N Benacerraf B Delafresnaye J F Charles C Thomas Publisher Springfield p 226–244 1957

Thomas L The Shwartzman phenomenon and other reactions produced by the endotoxins of Gram negative bacteria In 1 st Int Symposium on Immunpathology Ed Miescher P p 325 Schwabe Stuttgart, 1959

Thomas L & Good R A Effect of cortisone on the Shwartzman reactions The production of lesions resembling the dermal and generalized Shwartzman reactions by single injection of bacterial toxin in cortisone treated rabbits J exp Med 95 409–428 1952

Acta path microbiol scandinav 74 139-140 1968

BRIEF REPORT

IN VIVO EFFECTS OF ANTI ANGIOTENSIN II ON THE RENIN SYSTEM

Share of the Reninsystem in the Normal Blood Pressure Level and the Mechanism of the Changed Response to Renin in Nephrectomised Rats

By *Jens Bing* and *Knud Poulsen*

The share of the reninsystem in the normal blood pressure is unknown and little is known about the mechanism of the somewhat increased and markedly prolonged pressor response to renin which is found in nephrectomised animals (for litterature see *Page & Mc Cubbin* 1968) *Schaechtelin et al* (1964) found that this change is not connected with a prolonged persistence of renin in the blood and assumed that an accumulation of renin in the arterioles is responsible for the prolonged effect. The present study aims at elucidating these two problems by studying the effects of anti angiotensin II

Material and Methods

Normal female rats (about 200 g) or rats nephrectomised 21-24 hours before use were anaesthetized with amytal and injected with angiotensin II—amid (Hypertensin Ciba) and with hog renin using the standard preparation of Dr Haas (WHO Lab for Biol Stand) The anti angiotensin II was an immune plasma from rabbits 1 ml of which neutralised c 5-10 μg angiotensin II the association constant being 1×10^{9} l/M (*Poulsen* in press) Lysine 8 vasopressine Sandoz was given in doses of 1-3 International m Units and noradrenaline in doses of 20-80 ng

Results

Injection of 50 to 100 μl of the anti angiotensin II resulted in a most often transient lowering of the blood pressure (10-30 mm) in 9 of 13 normal rats but did not influence the blood pressure of 9 nephrectomised rats Such doses resulted in a subtotal or most often total unresponsiveness to 1 to 30 ng angiotensin and 1.4×10^{-3} GU renin in 13 *normal* rats (Fig 1A) while doses of 10 to 20 μl were found to result in distinct reduction of the response but not total unresponsiveness In contrast to this doses of 100 μl given to 13 *nephrectomised* rats made them only unresponsive to angiotensin while they still responded to renin The response had the same prolonged form but a maximal increase of only about 50 per cent of that found in non pretreated nephrectomised rats (Fig 1B) When anti angiotensin was injected into 7 nephrectomised rats after they had been given renin there was in most cases a decrease to a somewhat lower level of blood pressure which was still elevated over normal Further the response to i v injected angiotensin was lost while the animals were still able to react to a second dose of renin (Fig 1C) The typical prolonged pressor curve of nephrectomised animals was imitated by *continuous injection of angiotensin* into 1 normal and 5 nephrectomised rats in which subsequent injection of 100 μl anti angiotensin resulted in a blood pressure fall to the level found before the start of the injection (Fig 1D)

Controls 4 normal and 9 nephrectomised rats received 100 μl plasma from normal rabbits which in nearly all cases was found without effect on either blood pressure or pressor response to subsequently injected angiotensin and renin The anti angiotensin induced inhibition of the response to angiotensin and renin was specific in so far as the animals still responded to equipressor doses of vasopressin and noradrenalin

Received 29 vi 68 *from the University Institute for Experimental Medicine 71 Norre Alle Copenhagen 2100*

Supported by grants from "Kong Christian X s Fond" and Hjerteforeningen"

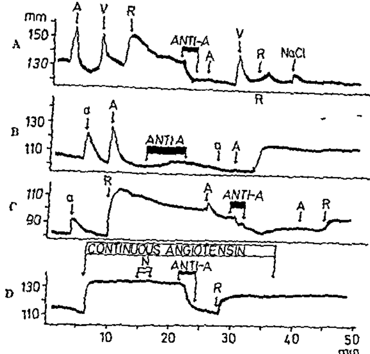


Fig 1

A In a normal rat the response to 4 ng angiotensin (A) and 1 4 × 10 ² Goldblatt Units (G U) renin (R) is abolished by 0 1 ml anti angiotensin II (Anti A) while the animal still responds to 2 5 mU vasopressine (V) NaCl = physiological saline

B In a nephrectomised rat the response to 1 2 and 2 4 ng angiotensin (a and A) is abolished by 0 1 ml anti angiotensin (Anti A) The renin response is typically prolonged, but the maximum height lower than in an untreated nephrectomised rat (dotted curve) with about the same initial sensitivity to angiotensin

C. In a nephrectomised rat pretreated with 1 4 × 10 GU renin (R) anti angiotensin changed the increased level to a lower but still increased plateau and made the rat unresponsive to a dose of 8 ng angiotensin (A) but still responsive to 1 4 × 10 GU renin

D The increased blood pressure level in a nephrectomised rat continuously injected with angiotensin 9 ng/min is uninfluenced by injection of normal rabbit plasma (N) but lowered to the initial level by anti angiotensin (Anti A) The animal is still able to react to renin (R) Cessation of the angiotensin injection does not influence the new increased level

Discussion and Summary

I Anti angiotensin injection resulted in a transient decrease in blood pressure in normal but not in nephrectomised rats indicating that the renin system plays a role in the level in normal animals II Anti angiotensin made normal rats unresponsive to both angiotensin and renin while nephrectomised rats were made unresponsive to angiotensin but still responded to renin with the typical prolonged response The maximum height of this response was lower however than in untreated nephrectomised rats Similarly a lower but still elevated level of blood pressure was most often found in nephrectomised rats when anti angiotensin II was injected after the renin injection These results support the idea that in nephrectomised rats some of the injected renin is accumulated (and in this way not eliminated as normally) in the arterioles a part of the angiotensin formed being in this way protected against anti angiotensin while another part is neutralised

References Page I H & J W McCubbin Renal Hypertension Chicago 1964
Schaechtelin G D Regoli & F Gross Am J Phys 206 1361–1364 1964

Acta path microbiol scandinav 74 141-142 1968

BRIEF REPORT

CHANGES IN THE ELECTROPHORETIC MOBILITY OF BP8 ASCITES TUMOUR CELLS AFTER TREATMENT WITH LYSOLECITHIN

By J N Mehrishi[1] D B Cater & F Hartveit[3]

Lysolecithin is a surface active haemolytic agent (*Robinson* 1961 *Klibansky & de Vries* 1963) *Munder Ferber & Fischer* 1965 described the effects of lysolecithin on cell membrane enzymes and *Butterworth & Cater* 1967 reported that lysolecithin reduced the oxygen uptake of tumour cells and leucocytes while characteristic damage to the tumour cell surface was seen in electronmicrographs (*Wilkinson & Cater* 1967) There is also a loss of K ions from mouse lymphocytes and BP8 ascites tumour cells (*Butterworth & Mehrishi* 1968) We therefore decided to measure the changes of electrophoretic mobility of BP8 cells after treatment with lysolecithin using the apparatus and technique described by *Seaman* 1965

Method

The tumour cells were washed 3 times in heparinised Hanks solution and resuspended in this medium Lysolecithin (ex egg lecithin) cryst (Koch Light Ltd Colnbrook England) after grinding in Hanks solution was added to the cell suspension and the electrophoretic mobilities at 25 C were determined within 5 to 7 minutes of its addition while the cells still looked intact under phase contrast microscopy All cell were used within 3 hours of harvesting from the mouse

TABLE 1

Quantitative Data of the Effects of Lysolecithin on BP8 Tumour Cells

Type of effect	Lysolecithin dose µg/10[6] cells		Reference
	Effect observed	50 % effect	
Inhibition of oxygen uptake	> 1 µg	6 4 µg	Butterworth Cater 1967
Changes in cell membrane seen in electronmicrographs	present with 2 µg	extensive with 4 µg	Wilkinson Cater 1967
K loss from cells	1 15 µg	1 15 µg	Butterworth Mehrishi 1968
Electrophoretic mobility reduced	0 6 µg	1 7 µg	This paper

Defined as half the maximum effect observed

Received 1 xii 68 from *Department of Radiotherapeutics Department of Pathology University of Cambridge England* and *Gade Institute Department of Pathology University of Bergen Norway*

[1] Supported by *the British Empire Cancer Campaign* [2] Gibb Fellow of the *British Empire Cancer Campaign* [3] Visiting Guest Worker Research Fellow *Norwegian Cancer Society*

Professor *J S Mitchell* is thanked for support

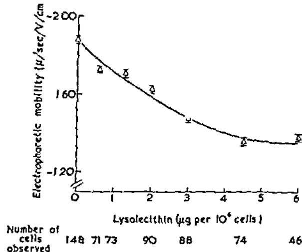


Fig 1

Results

The Figure shows the variation of the mean electrophoretic mobility (μ/sec/V/cm) ± S E.M with increasing doses of lysolecithin (μg/10⁶ cells) up to 6 μg

A decrease of 18 per cent was observed in the electrophoretic mobility of the lysolecithin treated tumour cells

Discussion

The quantitative data summarised in the Table suggests that electrophoretic mobility provides a sensitive index of change in cell surface This change could be of two types 1) *Alteration of chemical groups on the cell surface* *Mehrishi (1967)* studied the alteration of the electrophoretic mobility of saline washed BP8 cells with changes of pH and postulated the presence on the cell surface of both ionisable acid and basic groups Lysolecithin might alter the distribution or orientation of these surface groups thus leading to a change of electrophoretic mobility 2) The cell surface could be covered with products freed from the cells by damage inflicted by the lysolecithin Further work is planned (J N M) to investigate these possibilities both of which might be involved In view of the probable participation of lysolecithin in immune reactions *in vivo* (*Middleton & Phillips* 1963 1964 *Fischer* 1964 *Fischer et al* 1967) in the inflammatory response (*Cotran & Majno* 1964 *Munder et al* 1965) and its effect on tumour vessels (*Cater & Taylor* 1966 *Cater & Wallington* 1968) its action on tumour cells is noteworthy in connection with the physiopathology of tumour growth which may be dependent on a delicate balance between the immune and the inflammatory response related to tumour specific antigens (*Hartveit* 1968)

References 1 *Butterworth A F & Cater D B* Brit J Cancer 21 373–389 1967 —2 *Butterworth A E & Mehrishi J N* In press 1968 —3 *Cater D B & Taylor C R* Brit J Cancer 20 517–52? 1966 —4 *Cater D B & Wallington T B* Brit J exper Path 49 74–80 1968 —5 *Cotran R S & Majno G* Ann N Y Acad Sci 116 750–764 1964 —6 *Fischer H* Ann N Y Acad Sci 116 1063–1070 1964 —7 *Fischer H Ferber E Haupt I Kohlschütter A Modolell M Munder P G & Sonak R* Lysophosphatides and cell membranes Protides of the Biological Fluid Elsevier Amsterdam 15 175–184 1967 —8 *Hartveit F* Tidss norske legefor 88 1?18 1??? 1968 —9 *Klibansky C & de Vries A* Biochim biophys Acta 70 176–187 1963 —10 *Mehrishi J N* Brit Emp Cancer Campgn Ann Rep 4? ?5–?27 1967 —11 *Middleton E & Phillips C B* Nature (Lond) 198 758 760 1963 —12 *Middleton E & Phillips G B* J Lab clin Med 64 889–894 1964 —13 *Munder P G Ferber E & Fischer H* Z Naturforsch 20B 1048–1061 1965 —14 *Robinson N* J Pharm Pharmacol 13 321–354 1961 —15 *Seaman G V F* Cell Electrophoresis Churchill London 4–21 1965 —16 *Wilkinson P J & Cater D B* Ann Rep Brit Emp Cancer Campgn 45 230 1967

Acta path microbiol scandinav 74 143-144 1968

BRIEF REPORT

BETA HAEMOLYTIC STREPTOCOCCUS GROUP B CAUSING NEONATAL MENINGITIS

By *John Kvillingen*

Cases of neonatal infection caused by streptococci of the Lancefield's serological Group B have been reported during the past few years In our institute we have isolated streptococci Group B from two cases of neonatal meningitis and a short report on these cases may be of interest

Case No 1

On March 11th 1967 a sample of turbid spinal fluid was brought to the bacteriological laboratory Examination of a wet preparation using the phase microscope revealed cocci in chains and a stained smear showed Gram positive cocci By culture Growth of beta haemolytic streptococci The isolated strain was stored and not immediately examined serologically According to the very short case history accompanying the sample the patient a girl M W born on March 8th was admitted to the paediatric department of this hospital three days later with clinical symptoms Irritability and poor feeding

Case No 2

On March 26th 1967 a sample of turbid spinal fluid from another baby was received for bacteriological examination Beta haemolytic streptococci were isolated also in this case

According to the case history accompanying the sample from case no 2 the patient a girl S B M born on March 8th was admitted to the paediatric department on March 26th after one day of illness Fits No fever Moderately tense fontanelle

Serological examination classified the two strains of streptococci to the Lancefield Group B

The birth of the two babies on the same day in the same maternity hospital and isolation from both cases of the unexpected microbe indicated some further investigation

Case No 1

M W female infant born on March 8th 1967 at 4 a m Uncomplicated delivery 2 weeks after calculated term Birth weight 3370 g Mother healthy

Case No 2

S B M female infant born on March 8th 1967 at 8 a m Uncomplicated delivery at normal term Birth weight 3820 g Mother healthy

Both mothers lay in the same ward in the maternity hospital In case no 1 the midwife in charge of the delivery remained on duty up to 7 a m and during the night she undertook vaginal examinations of the mother in case no 2

Another midwife attending at 7 a m took charge of the delivery in case no 2

Comments

It is well known that streptococci Group B may be a commensal of the human female urogenital tract and examination of both of the mothers would have been of much interest Unfortunately we were able to obtain one sample only for

Received 8 viii 68 from the Bacteriological Laboratory Central Hospital Trondheim Norway

Confirmed by B Perch M D Statens Seruminstitutt Copenhagen

bacteriological examination namely from the mother of case no 2 21 days after delivery Streptococci Group B were not isolated by culture

The coincidence of neonatal meningitis caused by streptococci Group B in two cases of delivery under circumstances described above makes it most probable that one of the infants may be considered a victim of cross infection in the maternity ward

References Neonatal meningitis caused by streptococci Group B in one case *probably due to hospital cross infection* was also reported in PEDIATRICS OCTOBER 1966 No 4 part 1 For further references see those reports

Acta path et microbiol scandinav 4 145-160 1968

Institute of Pathology II (Head Prof S Falkmer MD)
University of Umeå Umeå, Sweden

AN ULTRASTRUCTURAL STUDY OF THE DISTRIBUTION OF HEAVY METALS IN THE PANCREATIC ISLETS AS REVEALED BY THE SULFIDE SILVER METHOD

By

ERIK PIHL

Received 1 1 68

Although it is well known light microscopically by histochemical and autoradiographical studies as well as by direct chemical assays that the pancreatic islet tissue of most vertebrates contain zinc (cf *Weitzel et al* 1953 *Davidson* 1958 *Voigt* 1959 *Bergman & Söremark* 1968) the ultrastructural distribution of this and other heavy metals in the islet parenchyma is still incompletely studied Preceding reports based on the application of ultrastructural modifications of the dithizone and sulfide silver procedures (*Okamoto & Kawanishi* 1966 *Kawanishi* 1966 *Pihl & Falkmer* 1967 *Pihl* 1967 1968) indicate that the metal is strictly localized to the secretion granules but a more comprehensive study on the distribution of heavy metals in various types of islet parenchymal cells is still lacking It was supposed that a comparative study of this kind performed on mammals with an islet tissue where histophysiological data are available might give some aspects on the poorly known physiological role of zinc in insulin and glucagon synthesis storage and release

The present investigation does not aim at covering all the main orders of mammals but only some of those species most commonly used in diabetes research By this however several of the important mammalian orders happened to be studied Thus from the Primates man was investigated from the Lagomorphs the rabbit from the Myomorph rodents the Chinese and Syrian hamsters the mouse and the rat from the Hystrichomorph rodents the guinea pig and the coypu (nutria) and from the Carnivores the cat and the dog Moreover one species from the Artiodactylan hoofed animals was included viz the reindeer mainly because the islet heavy metal of this animal has pre

This work was supported by grants from the *Swedish Medical Research Council* (Project No h67-1 X-718 0?) from the *Medical Faculty University of Umeå* and the *Nordic Insulin Fund*

viously been studied and another Odocoileinean animal of this main mammalian order the elk has been reported almost to lack heavy metals in the islet parenchyma (*Voigt* 1959)

MATERIAL AND METHODS

Fresh human pancreatic tissue was taken from three male patients at operation for gastric or renal carcinomas Likewise fresh pancreatic tissue was taken from adult healthy individuals of both sexes of those laboratory animals mentioned in the Introduction The animals were fed standard diets *ad libitum* and had free access to ordinary tap water Reindeer pancreas was obtained at the autumn slaughter in Lapland from seemingly sound and well nourished male animals In each species the number of animals used varied from two to ten

The Chinese hamsters used in this study originated from an inbred stock kept at the Institute of Pathology where some cases of spontaneous diabetes occur (cf *Boquist* 1968) However the animals used in this investigation were from a branch where no cases of overt diabetes had occurred since 5 generations Likewise no diabetes was known in the three human cases All other animals were from strains where no known heredity for diabetes existed

Tissue specimens were fixed in cold glutaraldehyde under continuous gassing with hydrogen sulfide as previously described (*Pihl* 1967 1968) In addition specimens were also fixed in cold 2 5 per cent glutaraldehyde for control purposes Dehydration and embedding in Epon or glycol methacrylate (GMA *Leduc & Bernhard* 1967) was carried out as previously described (*Pihl* 1967 1968) The electron microscopic procedures were also the same as in preceding reports Sections 1 μ thick were treated with the modified sulfide silver developer for 1½ to 4 hours and stained with toluidine blue The reaction was not considered negative until sections of islets from at least two blocks from three animals had been treated with a developer known to have the adequate colloidal properties and no reaction was seen after three hours Ultrathin sections were treated with the same developer for 20 to 30 min always using freshly prepared hydroquinone No cell type was considered negative until at least two different islets from each of two blocks had been developed and no reaction was seen even after reactivation i e reconversion to sulfides by also treating the ultrathin sections with sulfide (*Pihl* 1968) As controls sections 1 μ thick were used as well as ultrathin ones from glutaraldehyde fixed specimens which were treated with the sulfide silver developer as above

---

Fig 1

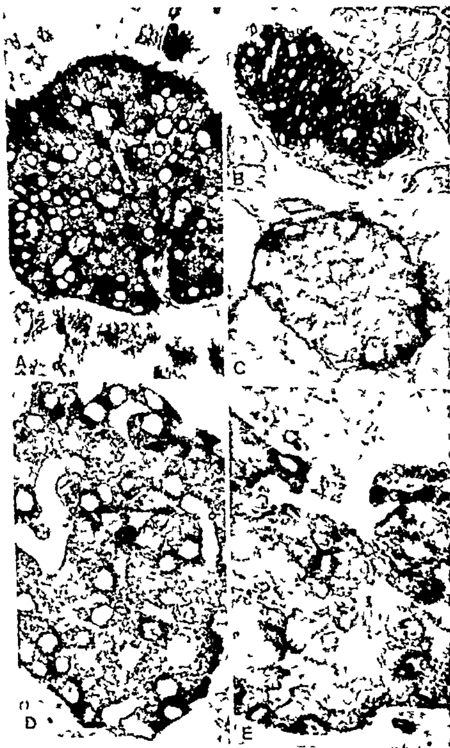
Fig 1a Photomicrograph from a human pancreatic islet The tissue was treated with hydrogen sulfide during fixation in glutaraldehyde dehydrated and embedded in Epon A section (1 μ thick) was treated with sulfide silver developer for 3 hours Two type of cells can be seen i e some which have taken up much silver and some which are more diffusely stained Nuclei have not reacted × 670

Fig 1b Syrian hamster pancreatic islet treated as in Fig 1a but embedded in GMA Physical development for 2½ hours The bulk of cells have reacted rather much with silver while the peripheral mantle of cells which in the electron microscope correspond to α cells are practically unstained × 280

Fig 1c A reindeer islet treated as in Fig 1a All islet cells have reacted only faintly with silver × 1170

Fig 1d Rat pancreatic islet treated as in Fig 1a and physically developed for 3 hours Clusters of peripheral cells (top and below) are more intensely stained than the central ones In the electron microscope the former are recognized as α cells and the latter as β cells × 1180

Fig 1e Dog pancreatic islet treated as in Fig 1a Some cells with a black perinuclear zone have reacted more with silver than the others but there is no characteristic distribution of the two types × 1220


A
B
C
D
E

Semiquantitative Result Evaluation

It was not considered possible to draw more than semiquantitative conclusions from the sulfide silver reaction in the pancreatic islets of various species Estimations pertain exclusively to the number of visible silver particles per preserved granule If the reaction did become positive only after "reactivation" it was designated (+) A + reaction refers to a small number of silver grains ++ a moderate number and +++ a large number

TABLE 1

The Distribution of Histochemically Reactive Heavy Metal (Zinc) in the Pancreatic Islets of Man and some Laboratory and Domestic Animals

Species	α -cells	β cells
Man	(+)	+++
Rabbit	++	+++
Chinese hamster	++	+++
Syrian hamster	+	+++
Mouse	++	++
Rat	++	++
Guinea pig	0	0
Coypu	(+)	traces
Cat	++	+++
Dog	(+)	+++
Reindeer	(+)	+

RESULTS

A strong sulfide silver reaction i e a large number of silver grains localized to secretion granules have been interpreted as indicating the occurrence of ample amounts of heavy metal The islet metal demonstrated was found almost exclusively in secretion granules of β and α cells and only in minute amounts in isolated lysosomes The findings in the various species investigated are given in Table 1 As a rule there

---

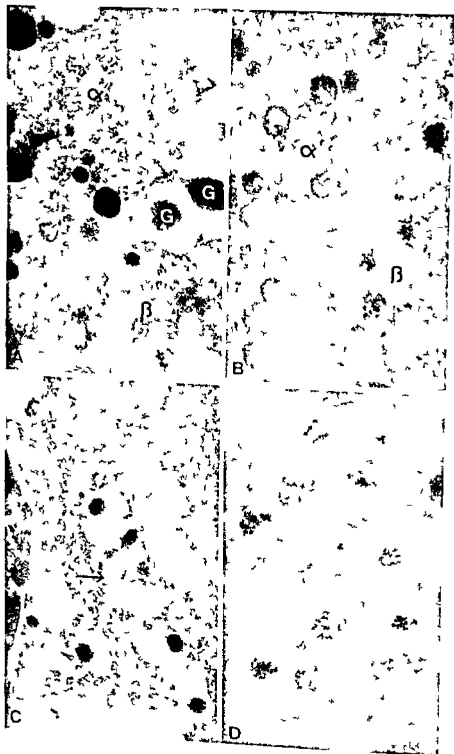
Fig 2

Fig 2a Electron micrograph from a human pancreatic islet treated as in Fig 1a The ultrathin section was physically developed for 20 min Only two β-cell granules (C) are fairly well preserved and show many small silver grains while the other β granules are in various stages of dissolution The α_2 granules (left) are well preserved Contrasted with uranyl acetate alone as all subsequent figures × 40 000

Fig 2b Rabbit pancreatic islet treated as in Fig 2a The β granules (right) are partly dissolved and show numerous small silver particles whereas the fairly well preserved α granules (left) show a moderate number × 44 000

Fig 2c Part of a Chinese hamster pancreatic islet β-cell treated as in Fig 2a This perinuclear field was chosen to show the best preserved granules one of which is partly dissolved (arrow) The rest are surrounded by wide halos and appear shrunken showing a fairly large number of silver grains While in other more affected parts only remnants of granules were seen the picture may suggest that these granules have passed through a stage of swelling × 32 000

Fig 2d Well preserved α granules from another Chinese hamster islet treated as in Fig 2a This was the maximum sulfide silver reaction seen in α granules of this species × 53 000


α
G
G
β
A
α
β
B
C
D

was little difficulty in identifying the two main types of islet cells. No data have been obtained so far regarding the occurrence of heavy metal in α_1 cells. The few agranular cells observed were sulfide silver negative.

Whereas α cells were generally rather well preserved when treated with hydrogen sulfide, most β cells showed a marked reactivity towards treatment with hydrogen sulfide. It was a common observation that β granules around the cell nucleus in the Golgi region and at the cell periphery were less affected by the gas. The phenomenon of granule dissolution and its possible implication on insulin release has been discussed elsewhere (*Falkmer & Pihl* 1968). A detailed account of our findings in the various animals follows below.

Man. Pancreatic islets reacted markedly with silver (Fig 1a), some cells being almost black while others were more faintly stained. In the electron microscope it was seen that the β cell granules had taken up much silver and had reacted rather intensely towards hydrogen sulfide (Fig 2a) with dissolution of many granules. In some islets the α granules yielded a weak reaction first after reactivation, while in others the reaction was weakly positive already after the initial gassing.

Rabbit. In this species β cell granules were also rather affected by the gas and gave a strong sulfide silver reaction (Fig 2b). The α cell granules, however, were fairly well preserved yielding a moderate reaction as in the cat.

Chinese hamster. The β granules were rather sensitive towards treatment with hydrogen sulfide and yielded a marked reaction (Fig 2c), whereas the α granules were but little affected with a moderate sulfide silver reaction (Fig 2d).

Syrian hamster. The β granules of the Syrian hamster were similarly affected by hydrogen sulfide as its Chinese relative and gave a fairly strong sulfide silver reaction. In one islet where the gassing was incorrectly carried out it was found that isolated cells which had come into contact with but little hydrogen sulfide to a large extent lacked preserved granules. Instead of granules there were numerous vacuoles surrounded by silver grains (Fig 3a). Adjacent cells were little affected (Fig 3b). Whether this phenomenon can be seen also in other species and may indicate different secretory stages will be further investigated. The α granules in the Syrian hamster pancreatic islets reacted moderately. As suggested by light microscopy (Fig 1b) these cells were found in the islet periphery showing a mantle of weakly stained cells.

Mouse. This species behaved rather similarly to the Syrian hamster. Thus β

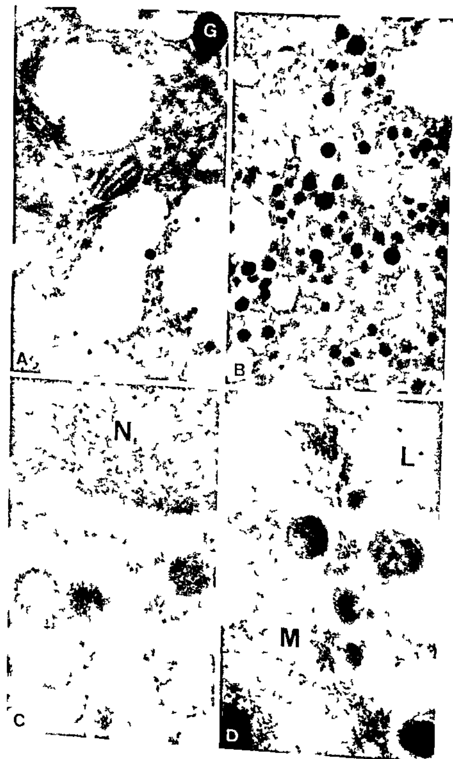
Fig 3

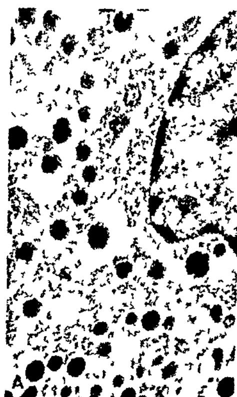
Fig 3a Part of a pancreatic islet β cell from a Syrian hamster immersed in glutaraldehyde bubbled through with hydrogen sulfide first at fixation. In isolated cells there were only single granules (G) left which had not reacted with the gas, whereas others seemed to have swelled and burst and left large vacuoles the periphery of which gave a sulfide silver reaction. Mitochondria are well preserved. CMA section. × 53 000

Fig 3b A β-cell from the same islet as in Fig 3a showing fairly good granule preservation. Apparently only a small amount of sulfide has reached the cell before fixation was complete, which is further suggested by the rather few and large silver grains. × 32 000

Fig 3 Mouse pancreatic islet β-cell treated as in Fig 2a. A few fairly well preserved granules are seen adjacent to the cell nucleus (N). These show a moderate number of silver grains. × 50 000

Fig 3d Part of a rat pancreatic islet β-cell treated with hydrogen sulfide for 10 min before fixation. This field was chosen from the best preserved part of the cell. The granules show a moderate number of silver grains, the lysosome (L) but few and the mitochondrion (M) none. × 50 000


G
A
B
N
L
M
C
D


A


C

D



Fig 5

Dog islet β cell treated as in Fig 2a The ultrathin section was further treated with sulfide (reactivation) Some granules are fairly well preserved in spite of the primary gassing while others show signs of dissolution The background of few small silver particles would be due to diffusion of heavy metal compounds during preparation × 45 000

granules were moderately well preserved with a moderate number of silver grains per granule (Fig 3c) while the α granules were almost intact and showed a moderate number of silver grains

Rat The β-cell granules yielded a moderate number of silver grains (Fig 3d) and were moderately affected by the gas When gassing had been carried out exclusively during fixation only isolated granules showed few and large silver precipitates The α granule reaction however could be much enhanced if gassing was performed before fixation

Guinea pig Both α and β cell granules (Fig 4a) were well preserved the sulfide silver reaction being uniformly negative even after reactivation

Coypu Also in this species all β granules were well preserved in spite of treat

---

Fig 4

Fig 4a Guinea pig pancreatic islet β cell treated as in Fig 3d with part of an α cell below In spite of gassing for 10 min before fixation the structure is fairly well preserved with only slight changes of the β granules No sulfide silver reaction × 31 000

Fig 4b Islet β-cells from a coypu (nutria) treated as in Fig 2a The rather electron dense β granules are no more affected here than in plain controls No sulfide silver reaction × 7 800

Fig 4c Cat islet β-cell treated as in Fig 2a The granules shown here are representative for those best preserved located perinuclearly as those of Fig 3c Yet they are rather affected by the gas and show numerous silver particles The nucleus (N) has a dentate appearance which was seen also in controls × 65 000

Fig 4d Part of an α-cell from the same block as in Fig 4c The well preserved granules show a moderate number of silver grains which for unknown reasons are much larger in one granule (arrow) × 39 000

ment with hydrogen sulfide (Fig 4b) In isolated cells a *weak sulfide silver reaction* was seen after reactivation of sections while most β cells remained negative Likewise the α granules yielded a weak reaction after reactivation

Cat Like in other animals investigated where the β cells contain much reactive metal it was difficult to obtain well preserved β granules in gassed tissue Those preserved showed a strong sulfide silver reaction with a fairly large number of silver grains (Fig 4c) Otherwise one could see that many β cells were almost devoid of preserved granules except for a few in the Golgi region and adjacent to nuclei (Fig 4c) and cell membranes The α cells of this species gave a moderately strong reaction with rather many small silver grains per granule (Fig 4d)

Dog In the light microscope one could already see that some islet cells gave a faint silver reaction whereas others without characteristic islet localization were almost blackened especially around nuclei (Fig 1c) In the electron microscope it was found that hydrogen sulfide led to dissolution of many granules of β cells Those preserved yielded a marked sulfide silver reaction confined to the disc like central structures (Fig 5) The α cells did not react until after reactivation and then rather weakly

Reindeer The reindeer islets were weakly positive without characteristic cellular distribution (Fig 1c) In the electron microscope the β granules were weakly positive and only moderately affected by the gas while the α granules gave a positive reaction only after reactivation

DISCUSSION

Evaluation of Results

Many factors may confuse assessment of the sulfide silver reaction as some pertinent variables are difficult to standardize At present there seems to be three major obstacles preventing more than semi quantitative conclusions to be drawn These are

1 The marked variation in colloid properties between different batches of gum Arabic In our hands the outcome of the reaction with different developers used on sections from the same block under standardized conditions varied from no outcome at all to a heavy layer over the whole pancreatic islet To some degree this could be compensated for by modifying the time of development after the light microscopical reaction on sections 1 μ thick

2 In order to obtain acceptable ultrastructure the conversion of tissue heavy metal to sulfide must take place as soon as possible and preferably at fixation (*Pihl* 1967) This would mean that only superficial parts of tissue come into contact with a surplus amount of SH ions before coagulation of proteins limits diffusion For this reason formaldehyde could not be used as combined fixative and vehicle of the gas (*Pihl* 1968) Thus working with pancreatic islet tissue it does not seem possible at present to compare equally gassed islets from time to time especially as the islets will be situated at various distances from the surface of the actual specimen

3 Hydrogen sulfide may be deleterious to the ultrastructure of islet cell granules containing much metal i e at least if pH and temperature are not strictly kept within narrow limits (*Pihl* 1968)

A way round the problems of points 2 and 3 would be to postpone treatment with hydrogen sulfide until later on ultrathin sections but evidently this entails a considerable risk of heavy metal diffusion during fixation and dehydration (*Pihl* 1968)

It would appear that the best structural and histochemical results are achieved if the initial gassing is carried out under strictly standardized conditions as described elsewhere (*Pihl* 1968) and only superficially situated islets are chosen for ultrathin sections. If one compares the sulfide silver reaction on sections developed immediately with those developed after "reactivation", valuable information may be obtained. The fact that the β granules of various species in contrast to the α granules seem to give a maximum reaction already after the initial gassing suggests that the heavy metal is bound to the latter granules in a different way, apparently harder. This holds true on the assumption that the glutaraldehyde is treated with hydrogen sulfide for one minute before the tissue is immersed and that continued gassing is performed as previously described (*Pihl* 1968). Thus α granules seem to give a maximum reaction only if a surplus amount of sulfide is administered through gassing before fixation or repeated on ultrathin sections.

Comparison with Previously Obtained Results

It is well known that no methods permit the simultaneous demonstration of heavy metal and a successful granule staining in pancreatic islet tissue though some conclusions may be drawn from the known topographical distribution of the various cell types in some animals, e.g. the occurrence of α cells in the islet periphery in the Myomorph rodents. In most animals light microscopical methods suggest that the heavy metal occurs within β cells (*Wolff & Ringleb* 1954). However there is evidence obtained with both the dithizone and the sulfide silver methods that at least in the rat more metal can be demonstrated in α cells (*Wolff & Ringleb* 1954, *Maske* 1955, *Voigt* 1959, *Yoshinaga & Shinji* 1963, *Weiss* 1965). *Muller & Guthert* (1960) could show that in the Syrian hamster the cells distributed around capillaries contained most metal and claimed that these were α cells. In the electron microscope we found that the α cells of this species were predominantly localized to the islet periphery, thus corresponding to the typical mantle islets of other Myomorph rodents. The α cells showed only a faint sulfide silver reaction or none at all (Fig. 1b), while the β granules contained fairly much metal (Fig. 3b). In this context it should be noted that fixation in alcohol as used in the sulfide silver method for paraffin sections leads to dissolution of β cell granules (*Lazarow* 1957). To deem from our results the same pertains to hydrogen sulfide also unless austere precautions are observed. Evidently dithizone has a similar effect (*Kawanishi* 1966). Thus it is difficult to tell from light microscopy whether one or the other cell type in a certain species contain most zinc knowing that α granules remain well preserved.

The heavy metal demonstrated with our method has almost exclusively been localized to secretion granules and only exceptionally to

lysosomes. Conceivably granule dissolution would cause the heavy metal to spread in all directions lowering the concentration locally. The stronger sulfide silver reaction around nuclei at the cell peripheries and adjacent to capillaries often seen in the various animals does not denote that these parts of the cells contain most metal but only that granule dissolution is less prominent in these regions. In no animals save in the nutria have we found more reactive metal in α_2 granules than in the β granules. Consequently we could not confirm *the previously made observations that the* α granules of rat and Syrian hamster contain more zinc than the β granules. We have also considered the fact that a surplus amount of sulfide has to be administered either before fixation or as reactivation in order to transform α granule metal to reactive sulfide, whereas β granules react already with small amounts of sulfide. The use of reactivation of sulfides thus does not solely ensure that sulfides oxidized during preparation are reconverted but also that a surplus amount of sulfide could reach heavy metal supposedly hard bound to protein in α granules. Evidently dithizone does not have sufficient affinity to make possible the demonstration of supposedly hard bound metal. Thus *Wolff & Ringleb* (1954) reported that the α cells of the dog contained no metal which is not in agreement with our results (Table 1) where α granules yielded a weak reaction after reactivation.

An important factor which must be considered when drawing conclusions as to which cell type that contains more metal in one or the other species is apparently the state of granulation, *i.e.* the nutritional status of the animal. *Maske* (1953) and *Wolff, Ringleb & Amann* (1955) showed that β cell zinc was stored during inanition and released at *hyperglycaemia, i.e. the metal content paralleled the degree of* β cell granulation. Working on the ultrastructural level however the sulfide silver reaction could be assessed as silver grains per granule. Thus differences in the degree of granulation may explain why light microscopy may show varying amounts of metal between different islets as well as between different animals of the same species. Likewise adrenalin may also lead to a decrease of β cell zinc (*Wolff, Ringleb & Amann* 1955). *Voigt* (1959) found no metal in three elks and rather little in a fourth animal. Likewise our reindeers showed rather few β cell granules although those found showed a clearly positive sulfide silver reaction. A positive sulfide silver reaction in reindeer islets has previously been described by *Voigt* (1959).

It is interesting to note that man and the carnivorous animals cat and dog have large amounts of reactive β cell heavy metal while the ruminant reindeer has considerably less. As to the Lagomorph rabbit this animal also has much β granule metal though possibly a little less than the carnivores showing less reactivity towards hydrogen sulfide. The Myomorph rodents generally had less reactive heavy metal in their β granules. On the ultrastructural level *Okamoto & Kawanishi* (1966)

and *Kawanishi* (1966) have reported that the β cells of rabbits and rats contain heavy metal also although no comparison was made and they do not mention whether the α cells also reacted or not

The Hystricomorph rodents were totally different from all other species hitherto investigated showing no β cell reactive metal as in the guinea pig or only insignificant amounts as in the coypu Whereas we have found no report in the literature as to pancreatic islet heavy metal in the coypu *Wolff & Ringleb* (1954) and *Voigt* (1959) have also reported that guinea pig islets give no heavy metal reaction or possibly sometimes only a trace reaction

Functional Significance of Islet Heavy Metal

Since *Okamoto* (1919) formulated his zinc theory about the pathogenesis of diabetes mellitus it has become more and more evident that zinc has a profound significance in islet metabolism although its role is largely unknown Insulin does not crystallize at physiological pH without heavy metals as zinc nickel cobalt or cadmium (*Scott* 1934) *Lazarow* (1957) thus concluded that zinc may play a role in the aggregation of insulin but adds that there is no evidence to suggest that this is a specific relationship as α cells may also contain large amounts of zinc *Maske* (1955) launched the view that there is an influence of this metal of the intracellular insulin depots This is in good agreement with the findings of *Wolff & Ringleb* (1954) that there is a close parallelism between the amount of histochemically demonstrable β cell zinc and the funcfional state of the cells Thus brief starvation which is known to lead to an increased number of β cell granules also led to more demonstrable zinc Reversely they found a decreased amount of β cell zinc when glucose was administered Thus there is much information to indicate that β cell zinc keeps insulin in a storable form within granules Apparently the metal is not necessary for the mere production of hormone (*Voigt* 1959)

The function of α_1 granule zinc is still more obscure These cells are known to contain and probably also produce glucagon (cf *Brolin & Hellerström* 1967) Little seems to be known about the interrelation between glucagon and metal within the α granules In contrast to zinc in β granules α cell zinc disappears after insulin administration and from starvation (*Wolff & Ringlel* 1954 *Weiss* 1965) The occurrence of one histidine residue in the glucagon molecule in contrast to two in the insulin molecule (*Bromer et al* 1957) might explain why also α granules seem to contain heavy metal in many species

In this context it might be of interest to note that in both guinea pigs and coypus none or only insignificant amounts of sulfide silver reactive heavy metal could be shown in β granules and that these granules were practically unaffected by hydrogen sulfide Conceivably these animals do not utilize zinc in their insulin metabolism It has

Ratte nach Adrenektomie und Insulinbelastung Endokrinologie 47 183-192 1965
Weitzel G Strecker F J Roester U Fretzdorff A M & Buddeke H Zink und Insulin im Pankreas von Knochenfischen Hoppe Seylers Z physiol Chem 295 83-106 1953
White A Handler P Smith E L & Stetten D W Principles of Biochemistry McGraw Hill New York Toronto London 1954
Wolff H & Ringleb D Histochemische Untersuchungen uber das Inselzink Z ges exp Med 124 236-256 1954
Wolff H P Ringleb D & Amann R Histochemische Untersuchungen über das Inselzink (II Histophotometrische Messungen) Z ges exp Med 126 390-416 1955
Yoshinaga T & Shinji Y Morphologische Untersuchungen des Langerhanschen Inseln des Pankreas Eine verbesserte Darstellungsmethode fur Zink Acta Histochem *16 337-342 1963*

and *Kawanishi* (1966) have reported that the β cells of rabbits and rats contain heavy metal also although no comparison was made and they do not mention whether the α cells also reacted or not

The Hystrichomorph rodents were totally different from all other species hitherto investigated showing no β cell reactive metal as in the guinea pig or only insignificant amounts as in the coypu Whereas we have found no report in the literature as to pancreatic islet heavy metal in the coypu *Wolff & Ringleb* (1954) and *Voigt* (1959) have also reported that guinea pig islets give no heavy metal reaction or possibly sometimes only a trace reaction

Functional Significance of Islet Heavy Metal

Since *Okamoto* (1949) formulated his zinc theory about the pathogenesis of diabetes mellitus it has become more and more evident that zinc has a profound significance in islet metabolism although its role is largely unknown Insulin does not crystallize at physiological pH without heavy metals as zinc nickel cobalt or cadmium (*Scott* 1934) *Lazarow* (1957) thus concluded that zinc may play a role in the aggregation of insulin but adds that there is no evidence to suggest that this is a specific relationship as α cells may also contain large amounts of zinc *Maske* (1955) launched the view that there is an influence of this metal of the intracellular insulin depots This is in good agreement with the findings of *Wolff & Ringleb* (1954) that there is a close parallelism between the amount of histochemically demonstrable β cell zinc and the funcional state of the cells Thus brief starvation which is known to lead to an increased number of β cell granules also led to more demonstrable zinc Reversely they found a decreased amount of β cell zinc when glucose was administered Thus there is much information to indicate that β cell zinc keeps insulin in a storable form within granules Apparently the metal is not necessary for the mere production of hormone (*Voigt* 1959)

The function of α_2 granule zinc is still more obscure These cells are known to contain and probably also produce glucagon (cf *Brolin & Hellerstrom* 1967) Little seems to be known about the interrelation between glucagon and metal within the α_2 granules In contrast to zinc in β granules α cell zinc disappears after insulin administration and from starvation (*Wolff & Ringleb* 1954 *Weiss* 1965) The occurrence of one histidine residue in the glucagon molecule in contrast to two in the insulin molecule (*Bromer et al* 1957) might explain why also α granules seem to contain heavy metal in many species

In this context it might be of interest to note that in both guinea pigs and coypus none or only insignificant amounts of sulfide silver reactive heavy metal could be shown in β granules and that these granules were practically unaffected by hydrogen sulfide Conceivably these animals do not utilize zinc in their insulin metabolism It has

recently been shown that guinea pigs made deficient in manganese show abnormal glucose tolerance (*Everson & Shrader* 1968). It seems possible that that metal could be demonstrated with our method should it occur within granules since manganese has a lower electrode potential (E_0 red.) than silver (*Lange* 1956). Whereas zinc is bound to the imidazol groups of insulin histidine located at the B_{10} position of most species (*Smith* 1966, 1968), guinea pigs and nutrias have at that position aspartic acid and glutamic acid respectively (*Smith* 1968), which amino acids are acidic (*White et al.* 1954) and would not be expected to bind zinc to the same extent.

From the present work with the sulfide silver method it seems plausible, at least as a working hypothesis for future work, that the metal plays some role in the regulation of insulin storage and release from intracellular granule dissolution (*Falkmer & Pihl* 1968). Seemingly hydrogen sulfide brings about a rapid imbalance of a homeostasis mechanism due to its interference with zinc from SH ions of the dissociated gas. Our interpretation of this phenomenon and its possible implication on insulin secretion under physiological conditions and various pathological states will be discussed elsewhere (*Falkmer & Pihl* 1968).

SUMMARY

By means of an ultrastructural sulfide silver method for demonstration of heavy metals, the pancreatic islet heavy metal (zinc) has been shown to occur in apparently large amounts in the β granules of man, dogs, cats, rabbits and Myomorph rodents, particularly Chinese hamsters. The β cell granules of the Hystrichomorph rodents, the guinea pig and the coypu, contained no or only insignificant amounts of reactive metal in isolated cells. A preceding observation that deer animals may have only small amounts of heavy metals in the β cells was confirmed so far that the reindeer just showed small amounts. A relationship was noted between the semiquantitatively estimated amount of β granule metal and the reaction of the β granules towards hydrogen sulfide which evoked dissolution of many granules. It was supposed that this reaction may have implications for the physiological role of zinc in the β granules.

It was found that moderate or small amounts of histochemically reactive heavy metal occurred also in the α_2 granules of man, rabbit, Myomorph rodents, carnivores and the reindeer. The only animal that completely lacked demonstrable heavy metal was the guinea pig, whereas its Hystrichomorph relative, the coypu, showed some α_2 granule metal.

No data were obtained as to the occurrence of heavy metals in α_1 cells. The few agranular cells observed seemed to lack histochemically detectable heavy metals.

Apart from the incidental finding of apparently minute amounts of heavy metal in isolated lysosomes of some β cells the occurrence of heavy metal in islet parenchymal cells was strictly localized to one kind of organelle only viz the secretion granules

REFERENCES

Bergman B & Sorémark R Autoradiographic studies on the distribution of zinc 65 in mice J Nutr 94 6 12 1968

Boquist L Morphology of the pancreatic islets of the non diabetic adult Chinese hamster *Cricetulus griseus* Light microscopical findings Acta Soc Med Upsal 72 331–344 1967

Brolin S E & Hellerström C Experimental diabetes research at the histological department in Uppsala Opusc Med 12 261–272 1967

Bromer W W Sinn L G Staub A & Behrens O K The amino acid sequence of glucagon Diabetes 6 234–238 1957

Davidson J Zinc content of toadfish (*Opsanus tau*) islet tissue Anat Rec *130* 403 1958

Everson G J & Shrader R E Abnormal glucose tolerance in manganese deficient guinea pigs J Nutr 94 89–94 1968

Fallmer S & Pihl E Structural lability of zinc-containing secretion granules of pancreatic β-cells after gassing with hydrogen sulfide Diabetologia vol 4 (no 4) In press 1968

Kawanishi H Electron microscopic studies on the secretory mechanism of pancreatic islet cells with particular reference to beta cells II Secretion of beta granules in islets of Langerhans particularly in association with intracellular reactive zinc under normal conditions during prolonged starvation and after administration of dithizone in rabbits Endocrinol Japon 13 384–408 1966

Lange N A Handbook of Chemistry Handbook Publishers Sandhusky Ohio 1956

La arow A Cell types of the islets of Langerhans and the hormones they produce Diabetes 6 222–233 1957

Leduc E H & Bernhard W Recent modifications of the glycol methacrylate embedding procedure J Ultrastruct Res 19 196–199 1967

Maske H Beobachtungen uber das Zink in den Langerhanschen Inseln des Pankreas und seine Beziehungen zur Inselfunktion Z Naturforsch 8 96–104 1953

Maske H Über die Beziehungen zwischen Insulin und Zink in der Langerhanschen Inseln des Pankreas Experientia 11 122–128 1955

Müller D & Güthert H Untersuchungen mit der Sulfidsilbermethode uber das Zink in den Langerhanschen Inseln des syrischen Goldhamsters Virchows Arch path Anat 333 97–100 1960

Okamoto K & Kawanishi H Submicroscopic histochemical demonstration of intracellular reactive zinc in β-cells of pancreatic islets Endocrinol Japon 13 305–318 1966

Pihl E Ultrastructural localization of heavy metals by a modified sulfide silver method Histochemie 10 126–139 1967

Pihl E Recent improvements of the sulfide silver method for ultrastructural localization of heavy metals J Microscopie vol 7 (no 4) In press 1968

Pihl E & Falkmer S Trials to modify the sulfide silver method for ultrastructural localization of heavy metals Acta Histochem 27 34–41 1967

Scott D A Crystalline insulin Biochem J 28 1592–1602 1934

Smith L F Species variation in the amino acid sequence of insulin Am J Med. 40 662–666 1966

Smith L F Species variation in the amino acid sequence of insulin Report of 6th Congr Int Diab Fed. Excerpta Med In press 1968

Voigt G E Untersuchungen mit der Sulfidsilbermethode an menschlichen und tierischen Bauchspeicheldrusen (unter besonderer Berucksichtigung des Diabetes mellitus und experimenteller Metallvergiftungen) Virchows Arch path Anat 332 295–323 1959

Weiss J Untersuchungen zur Frage der zentralnervosen Regulation der A Zellfunktion am Pankreas der Albinoratte I Das Verhalten des A Zellzinks der

Ratte nach Adrenektomie und Insulinbelastung Endokrinologie 47 183 192 1965
Weitzel G Strecker F J Roester U Fretzdorff A M & Buddecke E Zink und Insulin im Pankreas von Knochenfischen Hoppe Seylers Z physiol Chem 295 83-106 1953
White A Handler P Smith E L & Stetten D W Principles of Biochemistry McGraw Hill New York Toronto London 1954
Wolff H & Ringleb D Histochemische Untersuchungen über das Inselzink Z ges exp Med 124 236-256 1954
Wolff H P Ringleb D & Amann R Histochemische Untersuchungen über das Inselzink (II Histophotometrische Messungen) Z ges exp Med 126 390 416 1955
Yoshinaga T & Shinji Y Morphologische Untersuchungen des Langerhansschen Inseln des Pankreas Eine verbesserte Darstellungsmethode fur Zink Acta Histochem 16 337-342 1963

Acta path et microbiol scandinav 74 161–168 1968

The Department of Pathology II Karolinska Institutet Stockholm Department of Pathology Central Hospital Vasterås and Department of Histology University of Umeå Umeå 6 Sweden

THE ELECTRON MICROSCOPIC APPEARANCE OF THE GLOMERULAR LESIONS IN OBESE HYPERGLYCAEMIC MICE

By

A BERGSTRAND G NATHORST WINDAHL and B HELLMAN

Received 2 iv 68

The appearance in the light microscope of glomerular lesions in mice with the American variety of the obese hyperglycaemic syndrome has been described in a previous paper (*Nathorst Windahl & Hellman* 1964) The presence of nodular or more diffuse deposits of a hyaline glomerular material resulted in compression or even occlusion of the glomerular capillaries While the staining procedures revealed that the hyaline accumulation contained varying amounts of fat no amyloid could be demonstrated with histochemical methods

The present communication describes the examination of the glomerular lesions in the obese hyperglycaemic mice with regard to ultrastructural appearance and possible birefringence It was predicted that the use of the polarizing microscope and the electron microscope would not only give a more precise idea as to where the lipohyaline deposits are located but also help in evaluating the extent to which they are related to amyloidosis or to experimental or spontaneous diabetic glomerulosclerosis

MATERIALS AND METHODS

Six obese hyperglycaemic mice (ob ob) of both sexes from the strain originating from R B Jackson Memorial Laboratories Bar Harbor Maine U S A were studied All animals were more than 12 months old and their kidneys contained the lipohyaline glomerular lesions characteristic of ageing obese hyperglycaemic mice

Small pieces from one of the kidneys were fixed in ice-cold buffered osmium tetroxide dehydrated in acetone and embedded in Vestopal W The osmium fixed material was either cut into 1 μ thick sections and stained with safranine (*Schantz & Schecter* 1965) for light microscopy or cut into thin sections and stained with

This investigation represents part of a project supported by the *Medical Research Council of the Swedish Life Offices* and the *Swedish Medical Research Council* Our thanks are also due to *Diego Carlstrom* M D Department of Biophysics Karolinska Institutet Stockholm for generous help and advice with the polarizing microscope



Fig 1

Light microscopic picture of a severely damaged glomerulu The lipohyaline masses compress and partially occlude the glomerular capillaries The deposits are located inside the basement membrane which is well preserved Osmium fixed tissue embedded in Vestopal and stained with Safranine 1 000 ×

uranyl acetate for examination in an electron microscope (Siemens Elmiskop I) The other kidney was fixed in 10 per cent neutral formalin and embedded in paraffin After staining with van Gieson or Congo red 5 μ thick sections of the paraffin embedded material were examined in the light and polarizing microscopes Frozen kidney sections were also prepared and stained for lipids with Scarlet red and Sudan black B

RESULTS

Light and polarization microscopy Examination of the frozen or paraffin embedded kidney sections in the light microscope made it evident that diffuse and nodular lipohyaline glomerular changes were present There was also weak staining with Congo red Light microscopic examination of the Vestopal embedded kidney material revealed severe destruction of most glomeruli with large deposits compressing the capillaries The capillary basement membrane was however well preserved (Fig 1) In the polarizing microscope the lipohyaline masses displayed weak focal birefringence

Electron microscopy Survey pictures o whole glomeruli (Fig 2) indicated that the lipohyaline masses wer lized to the areas and to the endothelial side of the ent membr



Fig 2

Survey picture of glomerulus The lipohyaline masses are concentrated to the mesangial areas The nuclei of the mesangial cells and probably also those of the endothelial cells inside the deposit are slightly pycnotic The basement membrane is strongly osmiophilic and distinctly separated from the deposits VP = vascular pole JC = juxtaglomerular granulated cell MD = macula densa Electron micrograph as in the following pictures 1 700 ×



Fig 3

Part of a glomerular capillary End = endothelial cell Ep = epithelial cell M = remnants of mesangial cell cytoplasm The fibrillar structure of the lipohyaline mass is very prominent 10 000 ×

capillary walls The basement membrane was well preserved and had a higher electron density than the lipohyaline masses No apposition of a basement membrane like substance or thickening of this membrane was found The hyaline deposits had a well defined structure and consisted either of partly parallel fibrils or rounded strongly osmiophilic globules surrounded by finely granulated material containing short fragments of fibrils (Figs 3–5) The fibrils were about 70 A broad The globules were scattered among the lipohyaline masses

---

Figs 4–5

Fig 4 Capillary wall A partly parallel (lower right) partly irregular (centre) organization of the fibrils is seen in the deposits together with scattered electron dense globules A network of fine fibrils is also observed on the endothelial (lower) side of the basement membrane (B) E = endothelial cell Ep = epithelial cell 25 000 ×

Fig 5 High magnification of a capillary wall Ep = epithelial cell D = deposit Numerous free ribosomes and slightly dilated cisterns of the endoplasmic reticulum can be seen in the epithelial cell cytoplasm The electron dense globules are concentrated close to the basement membrane The deposit also contains fragments of fibrils 50 000 ×


Ep
E
Ep
B
D

but displayed a marked concentration to the endothelial side of the basement membrane The electron dense globules were slightly irregular in size with an average diameter of *300–400 A (Fig 5)*

The endothelial and mesangial cells were destroyed cytoplasmic remnants and pycnotic nuclei being observed in the lipohyaline masses (Fig 3) The capillary epithelial cells were also affected to some extent probably due to the compression of the capillaries and protein leakage *through the capillary walls The foot processes appeared on the other* hand to be well preserved and the granulated endoplasmic reticulum *was abundant with slightly widened cisternes*

DISCUSSION

Spontaneous glomerular lesions with thickening of the capillary walls and hyaline deposits in the mesangium have been described in ageing mice of different strains (*Gorer* 1940 *Gude & Upton* 1962) A more *detailed evaluation of these studies* is hampered by the fact that they have not included electron microscopy Although the lesions mentioned have some resemblance to the present observations it should be pointed out that the obese hyperglycaemic mice do not display any obvious tendency to glomerular fibrosis However it is possible that the diabetic state of the obese hyperglycaemic mice accelerates an otherwise inconspicuous ageing process (*Nathorst Windahl & Hellman* 1965) *Guttman & Kohn* (1963) have for example found spontaneous glomerular lesions in mice which were markedly enhanced by radiation

Dunn (1944) and *Thung* (1957) have described a type of spontaneously appearing senile amyloidosis in mice Judging from these authors description the amyloid lesions were very similar to or identical with the glomerular lesion observed in the obese hyperglycaemic mice In fact the lipohyaline material has many features in common with human or experimental *amyloid a distinct fibrillar* structure and accordingly a weak optical birefringence which was *enhanced after staining* with Congo red The large amounts of fat and the small electron dense globules which probably contain lipids are however not characteristic of true amyloid Furthermore the location of the lipohyaline masses differs from that of amyloid which is known to precipitate on both sides of the lamina propria of the basement membrane and to gradually dissolve *the membrane* (*Cohen & Calkins* 1959 *Caesar* 1960 *Bergstrand & Bucht* 1961)

A comparison with human diabetic glomerulosclerosis is also of interest in view of the fact that the kidney lesions occur in animals with hereditary diabetes The lipohyaline glomerular masses have the same location in the obese hyperglycaemic mice as the hyaline deposits found in globular or diffuse human glomerulosclerosis (*Bergstrand & Bucht* 1959 *Farquhar et al* 1959 *Dachs et al* 1964) Fat is occasionally observed in the latter deposits but never to the same extent as in the

glomerular lesions of the obese hyperglycaemic mice Furthermore a distinct fibrillar or globular structure cannot be seen in the deposits of human diabetic glomerulosclerosis which have the same appearance as the basement membrane It may therefore be concluded that the glomerular lesions in the obese hyperglycaemic mice differ from those in human diabetes

Glomerular lesions are frequently observed in animals with experimental diabetes (*Lukens & Dohan* 1946 *Mann et al* 1951 *Bloodworth & Hamwi* 1955 *Hartroft* 1955 *Greenberg* 1962 *Bloodworth* 1963) Nodular glomerular lesions induced by cortisone have also been described and compared with human diabetic glomerulosclerosis (*Janes & Sommers* 1957 *Rammer et al* 1967) None of these glomerular changes seems to be identical with those found in the obese hyperglycaemic mice

The lipohyaline deposits are mainly and probably primarily localized to the mesangial cells which are the main target both in inflammatory and immunologic reactions of the glomeruli Present observations sustain the previous suggestion (*Bergstrand & Bucht* 1959 1961) that the mesangial cells derive from pericytes and whether considered from a phylogenetic or a functional point of view are homologous to the reticulum cells of the liver spleen and lymphatic organs

SUMMARY

Prominent glomerular lesions have been observed in ageing obese hyperglycaemic mice Electron microscopic studies revealed that lipohyaline glomerular deposits were localized both to the mesangial areas and to the capillary walls between the basement membrane and the endothelial cells The lipohyaline masses displayed a weak focal birefringence in the polarizing microscope While the glomerular lesions of the obese hyperglycaemic mice differ in several respects from diabetic glomerulosclerosis human amyloid and experimentally induced amyloid in animals they appear to be similar to what has previously been termed senile amyloidosis in mice

REFERENCES

Bergstrand A F & Bucht H The glomerular lesions of diabetes mellitus and their electron microscopic appearance J Path Bact 77 231–242 1959

Bergstrand A F & Bucht H Electron microscopy and renal function in amyloidosis of the kidney J Path Bact *81* 495 503 1961

Bloodworth J M B Diabetic microangiopathy Diabetes *12* 99–114 1963

Bloodworth J M B & Hamwi G J Histopathology of experimental glomerular lesion simulating human diabetic glomerulosclerosis Am J Path *31* 167–187 1955

Cossar R Die Feinstruktur von Milz und Leber bei experimenteller Amyloidose Z Zellforsch 5? 653–673 1960

Cohen A S & Calkins E Electron microscopic observations on a fibrous component in amyloid of diverse origins Nature Lond *183* 1202–1203 19 9

Dachs S Churj J Mautner W & Grisham E Diabetic nephropathy Am J Path *44* 155–168 *1964*
Dunn T B Relationship of amyloid infiltration and renal disease in mice. J Nat Cancer Inst 5 17–28 1944
Farquhar M C Hopper Jr J & Moon H D Diabetic glomerulosclerosis Electron and light microscopic studies Am J Path 35 721 754 1959
Gorer P A Renal lesions found in pure lines of mice J Path Bact *50* 25–30 1940
Greenberg S R Glomerular changes in chronic alloxan diabetes Arch Path *73* 263–273 1962
Gude W D & Upton A C Histologic study of spontaneous glomerular lesions in ageing RF mice Am J Path *40* 699–710 1962
Guttman P H & Kohn H I Age at exposure and the acceleration of intercapillary glomerulosclerosis in mice Lab Invest *12* 250–256 1963
Hartroft W S Fat emboli in glomerular capillaries of choline deficient rats and patients with diabetic glomerulosclerosis Am J Path *31* 381–397 1955
Janes R G & Sommers S C Glomerular alterations in kidneys of rats treated with desoxycorticosterone Arch Path *64* 58–62 1957
Lukens F D W & Dohan F C Experimental pituitary diabetes of five years duration with glomerulosclerosis Arch Path *41* 19–24 1946
Mann G V Goddard J W & Adams L The renal lesions associated with experimental diabetes in the rat Am J Path *27* 857–869 1951
Nathorst Windahl G & Hellman B Lipohyalin glomerular lesions in ageing obese hyperglycemic mice Med Exp *10* 67–71 1964
Rammer L Nathorst Windahl G & Boberg J Cortisone nephropathy in the rabbit The effect of heparin on the morphology plasma lipid levels and plasma lipoprotein lipase activity Acta Path *71* 161–172 1967
Schant A & Schecter A Iron hematoxylin and Saffranin as a polychrome stain for epon sections Stain Technol *40* 279 282 1965
Thung P J The relation between amyloid and ageing in comparative pathology Gerontologia *1* 234–254 1957

Acta path et microbiol scandinav 74 169 178 1968

The University Institute for Pathological Anatomy and The Department of Surgical Research Rikshospitalet Oslo Norway

THE *IN VIVO* UPTAKE OF OESTRADIOL 17β BY HORMONE RESPONSIVE AND UNRESPONSIVE BREAST TUMOURS OF THE RAT

By

STEN SANDER and ARNE ATTRAMADAL

Received 5 iv 68

In some breast tumours induced in rats by 7 12 dimethylbenz(a) anthracene (DMBA) growth is dependent on the presence of ovarian hormones (5 9 19)

In other animals however the tumour growth will continue unaffected by ovariectomy (4 19)

The histological structure is not markedly different in these two types of tumour (17)

Tritiated oestradiol 17β administered to rats with DMBA induced breast tumours is taken up and accumulated in the tumour (11) The investigation published by *Mobbs* (14) indicates that there exists a correlation between the uptake of oestradiol in a tumour and the response to ovariectomy If such a correlation exists it might be of importance for the prognosis of individual tumour response In the present study hormone responsive and hormone unresponsive DMBA tumours are compared with regard to

1) Measurement of uptake capacity for oestradiol 17β and
2) Localization of oestradiol ^3H in various components of the tumour tissue

MATERIALS AND METHODS

Non inbred female Wistar rats were used (bred by Møllegaard A L Denmark) At the age of 50 ± 2 days each rat was given 4 mg of 7 12 dimethylbenz(a)anthracene by intravenous injection (5 mg per g in a special 15 per cent fat emulsion Upjohn Companies Kalamazoo Michigan) A second dose of 4 mg was injected three days later (6)

The rats were examined twice weekly for tumours Palpable tumours appeared

This work has been supported by *Norsk Forening til Kreftens Bekjempelse* Oslo

The dimethylbenzanthracene (DMBA) was generously provided by the *Upjohn* Company

The authors wish to express their appreciation to Mrs *Jane I[illegible]sen* for her skilled technical assistance

from 5 to 50 weeks after administration of the carcinogen Growth of the tumours was assessed by measuring two diameters with calipers through the skin (19) The average of the two diameters was used as a measure of the tumour size Only animals with growing tumours were used

When the average diameter was from 12-16 mm oophorectomy was carried out The tumours were then measured every second day 5 8 days after oophorectomy the tumours were classified in two groups

Group 1 Tumours in regression The average diameter decreased more than 4 mm

Group 2 Tumours which continued to grow

After classification of the tumours the rats were given 0 2 μg (6 7 ^3H) oestradiol 17β/100 g body weight by intramuscular injection (New England Nuclear Corporation specific activity 166 $\mu C/\mu g$) The oestradiol-^3H solution was prepared and purity checked as previously described (16) The rats were killed by decapitation ½ 2 and 4 hours after injection Tiny pieces of tumour tissue were taken out immediately for autoradiographic examination (1 16) Control sections from non radioactive tumour tissue were used for determination of unspecific labelling (15) Other portions of the tumours were fixed in 4 per cent formaldehyde Sections were cut at 5 μ from paraffin blocks and stained with haematoxylin and eosin

Tumour tissue and skeletal muscle were taken out for measurement of radioactivity In 8 cases the uptake of label in pituitary gland uterus and adrenal glands was also studied

After excision of the tumours possible necrotic areas were removed The tissue was left in 4 ml 0 5 N NaOH over night and subsequently treated in a Potter Elvehjem homogenizer fitted with a Teflon pestle A second treatment was given by ultrasonic disintegration for 3 minutes (MSE 100 W Ultrasonic Disintegrator) At this stage aliquots were taken for liquid scintillation counting and protein determination

Radioactivity was estimated in a liquid scintillation system Naphthalene 80 g formic acid 10 g P P O (2 5 diphenyl oxazole) 5 g Dimethyl P O P O P (1 4 bis 2 (4 methyl 5 Phenyloxazolyl) Benzene) 0 05 g in ethanol 230 ml Dioxan 385 ml and xylol 385 ml

24 hours after preparation the samples were counted in a liquid scintillation spectrometer (Nuclear Chicago Mark I model 6860) In general at least 10 000 counts were recorded. All counts were corrected for background activity quenching and counting efficiency The degree of quenching was calculated with external standard (2) Protein determinations were performed on diluted aliquots by Lowry s method (13) Concentration of radioactivity in the tissues is expressed as disintegrations per min/mg protein The tumour muscle ratio of radioactivity was calculated for each tumour

RESULTS

A total of 90 injected rats yielded 43 breast tumours in 41 rats Nine tumours were fibro adenomata or a mixed type adenomata which did not respond to oophorectomy In some of the fibro-adenomata proliferation of the epithelium suggested potential malignancy

The 34 remaining tumours were adenocarcinomata After oophorectomy 23 of these carcinomata showed regression as indicated by a decrease in the average diameter (12 of these were used for the present study) Eleven tumours continued to grow after oophorectomy

The histological structure varied Some tumours showed papillary tubular or acinar forms while sheets of more uniform and anaplastic cells were observed in others (Figs 1 and 2) Frequently the same tumour showed mixtures of different morphological types Most of the tumours were well encapsulated but in some cases tumour infiltration

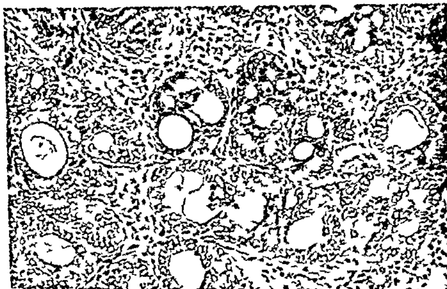


Fig 1

Luminated glandular structure in a hormone responsive tumour stained with haematoxylin and eosin × 440



Fig 2

Variation in the histological structure of a hormone unresponsive tumour stained with haematoxylin and eosin × 440

of the capsule and surrounding tissue was observed No metastases were found

The histological structure of the tumours in the two groups was not essentially different Changes believed to represent signs of regression

were observed in most tumours from group 1. Flattening of the epithelium with increase in the lumen of the acini was frequently seen during the process of regression. The concentration of radioactivity in the two tumour groups is shown in Table 1. Although there is considerable variation between the tumours within the same group, the tumour: muscle ratio was distinctly higher in hormone responsive tumours (group 1) compared to unresponsive tumours (group 2).

TABLE 1

Concentration of Radioactivity in Breast Tumours and Skeletal Muscle After the Injection of 0.2 µg (6,7-³H)Oestradiol/100 G Body Weight

Animal no	Response to oophorectomy	Time after injection	Radioactivity in Dpm/mg protein in: Tumour	Muscle	Ratio Tumour: Muscle
D 7	Responsive	½ hour	1 510	237	6.4
D 8	Responsive		1 480	295	5.0
D 19	Responsive		2 650	492	5.4
D 20	Responsive		1 380	398	3.5
D 1	Unresponsive	½ hour	464	252	1.8
D 5	Unresponsive		427	270	1.6
D 6	Unresponsive		545	219	2.5
D 30	Unresponsive		1 050	246	4.3
D 12	Responsive	2 hours	1 500	127	11.8
D 13	Responsive		1 460	85	17.2
D 27	Responsive		1 090	130	8.4
D 28	Responsive		1 600	120	13.3
D 2	Unresponsive	2 hours	472	76	6.2
D 3	Unresponsive		347	93	3.7
D 4	Unresponsive		446	82	5.4
D 14	Unresponsive		305	113	2.7
D 9	Responsive	4 hours	1 810	95	19.1
D 15	Responsive		680	70	9.8
D 16	Responsive		799	72	11.1
D 17	Responsive		8[illegible]5	57	14.5
D 10	Unresponsive	4 hours	268	64	4.2
D 11	Unresponsive		6[illegible]0	102	6.2
D 18	Unresponsive		323	68	4.7

The calculated ratios fell into two separate populations correlated to group 1 and group 2. It will be seen (Fig. 3) that in group 1 the tumour: muscle ratio increased from ½ h up to 4 h after injection of the labelled oestradiol. This means that the concentration of radioactivity in muscle tissue decreased rapidly during this period while the tumour tissue retained a major fraction of the accumulated radioactivity.

On the other hand, the ratio tumour: muscle in group 2 is only slightly higher 2 h and 4 h after injection. Thus the tumours in this

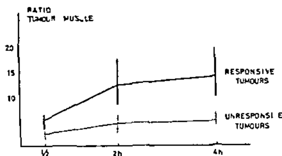


Fig 3

The ratio of concentration of radioactivity in tumour muscle tissue is given for group 1 and group 2 tumours Each point on the curve represents the median value of 4 tumours The range of the observations is indicated by the vertical bars

group do not retain the labelled oestradiol to the same extent as tumours in group 1

The concentration of radioactivity in adrenal gland uterus and pituitary gland was not significantly different in the two groups

Autoradiography revealed a marked accumulation of radioactivity in tumours from both groups In agreement with the results of the measurements the concentration of silver grains seemed to be higher in group 1 tumours than in group 2 The tumour cells were more heavily labelled than those of the stroma in all sections The distribution of the label was essentially the same at all observed intervals after the administration of oestradiol ³H The majority of silver grains seemed to be situated in the tumour cell nuclei (Fig 4) A striking number of grains were however found close to the nuclear membrane (Fig 5) Accordingly with the use of light microscopy it is difficult to decide whether the label is on the cytoplasmic or the nuclear side Some grains were also present in the cytoplasm of the tumour cells distinctly separated from the nuclear membrane (Fig 6)

DISCUSSION

Adenocarcinoma of the breast in rats can be divided into hormone responsive and hormone unresponsive groups depending on the effect of oophorectomy So far satisfactory criteria are not available for prediction of the individual response of a tumour The histological structure does not permit conclusions as to which tumour will regress and which will continue to grow after oophorectomy The local uptake of oestrogens in tumour tissue may yield valuable information in this respect (3)

In our study measurement of radioactivity as well as autoradiography revealed that tumours from both groups accumulate the labelled oestradiol There was however a distinct difference in the uptake capacity of the tumours The hormone responsive tumours had a



Fig 4



Fig 5

Figs 4–6

Distribution of tritium labelled estradiol in DMBA induced breast tumours Phase contrast photomicrographs of autoradiographs exposed for 8 weeks at —30 C. Unstained sections 0 5 μ thick Some of the silver grains in the stripping film appear white due to focus in a slightly different plane

Fig 4 Distinct labelling of the cell nuclei in a hormone responsive tumour × 2 600

Fig 5 Silver grains are frequently located near the nuclear membrane × 2 600

Fig 6 Silver grains are also seen in the cytoplasm of tumour cells Hormone unresponsive tumour × 3 200



Fig 6

higher uptake of oestradiol compared to the hormone unresponsive tumours This observation is in agreement with the findings published by Mobbs (14)

In her study the growth response to ovariectomy was assessed following biopsy of the tumours Biopsy makes it possible to measure the uptake capacity before the effect of ovariectomy is introduced However any surgical intervention may influence tumour growth by interference with the local blood supply or by causing an inflammatory reaction

In our study the tumour was left intact and the operative procedure was restricted to oophorectomy Thus the possible variations in oestradiol uptake due to the oestrus cycle were minimized

We examined the uptake capacity after oophorectomy when the hormonal balance of the host was changed and regression induced in some of the tumours Thus the possibility exists that the observed difference in uptake capacity was due to regression in group 1 tumours Therefore the examinations were carried out before the process of regression in group 1 tumours was advanced

Our findings indicate that the oestradiol uptake depends on individual characteristics of the tumour The observed correlation between castration response and uptake capacity in these tumours is consistent with the results published by *Mobbs* (14)

Fibro adenomata is known to occur in rats whether or not they are treated with carcinogens although the incidence may be influenced by the carcinogen (9) The fibro adenomata is not discussed in this study The histology of DMBA induced breast tumours has been described in

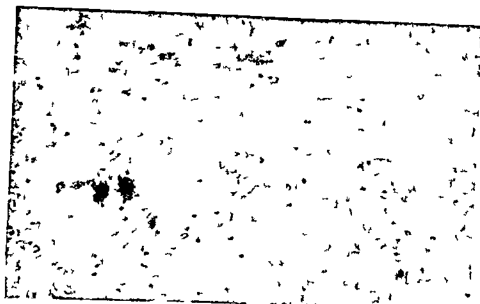


Fig 4

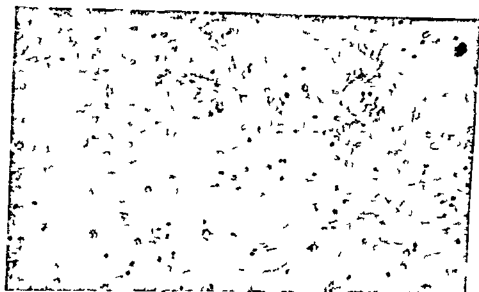


Fig 5

Figs 4–6

Distribution of tritium labelled oestradiol in DMBA induced breast tumours Phase contrast photomicrographs of autoradiographs exposed for 8 weeks at —30 C Unstained sections 0.5 μ thick Some of the silver grains in the stripping film appear white due to focus in a slightly different plane

Fig 4 Distinct labelling of the cell nuclei in a hormone responsive tumour × 2 600

Fig 5 Silver grains are frequently located near the nuclear membrane × 2 600

Fig 6 Silver grains are also seen in the cytoplasm of tumour cells Hormone unresponsive tumour × 3 900

7 12 dimethylbenz(a)anthracene (DMBA) and only growing adeno carcinomata were examined

Following oophorectomy the tumours were classified as hormone responsive or unresponsive The uptake capacity of the tumours was determined ½ 2 and 4 hours after intramuscular injection of oestradiol-³H In agreement with previous investigations it was found that hormone responsive tumours accumulate distinctly more oestradiol than unresponsive tumours Thus there seems to be a significant correlation between the response to oophorectomy and the uptake capacity for oestradiol

Autoradiography revealed a marked labelling of tumour cells in hormone responsive as well as unresponsive tumours The labelled oestradiol was found mainly in the nuclei of the tumour cells The distribution of silver grains was not apparently different in the two tumour groups Quite some silver grains were also present over the cytoplasm distinctly separated from the nuclear membrane The results are discussed with reference to cell fractionation studies and previous reports on the uptake of oestradiol in DMBA induced breast tumours

REFERENCES

1 *Attramadal A* Z Zellfors h Mikroskop Anat To be published
2 *Birlle L A* Determination of liquid scintillation counting efficiency by pulse height shift Int J appl Radiat *8* 1 6 1960
3 *Fi|a l J Clas J R F & Irvine W T* Studies with tritium labelled hexoestrol in advanced breast cancer Lancet *2* 796–798 1961
4 *Gru nstein M Meran e D R Thatcher D & Shimkin M B* Carcinogenic effects of intragastric 3 methylcholanthrene and 7 12 dimethylbenz(a) anthracene in Wistar and Sprague Dawley Rats J Nat Cancer Inst *36* 483–502 1966
5 *Huggins C Grand L & Brillantes F P* Mammary cancer induced by a single feeding of polynuclear hydrocarbons and its suppression Nature *189* 204–207 1961
6 *Hugg ns C Gra d L & Fukunishi R* Aromatic influences on the yields of mammary cancers following *administration of 7 12* dimethylbenz(a) anthracene Proc Natl Acad Sci *51* 737–743 1964
7 *Jensen E V & Ja obson H I* Basic guides to the mechanism of estrogene action Recent Progr Hormone Res *18* 387–414 1962
8 *Jensen E V D Sombre E R & Jungblut P W* Estrogen Receptors in Hormone responsive Tissues and Tumor Endogenous Fa tor Influen ing Host Tumor Balance *The University of Chicago Press* Chicago 1967 p 15 30
9 *Jull J W* The effect of hormonal environment and genetic constitution on mammary tumor induction in rats by 7 12 dimethylbenz(a)anthracene C n er Res *26* 2368– 373 1966
10 *Jungblut P W Hat el I DeSombre E R & Jensen E V* Ub r hormon Re ceptoren Di oestrogenbindenden Prinz pien der Erfolgesorgane Wirk ungsmechanismen der Hormone *Springer Verlag* Berlin 1967 p 58 8?
11 *King R J B Cowan D M & Inman D R* The uptake of (6 7 ³H) oestradiol b dimethylbenzanthracene induced rat mammary tumours J Endocrin *32* 83 90 196
12 *King R J B & Gord n J* The association of (6 7-³H) oestradiol with a nuclear protein J Endocr n *39* 533–542 1967
13 *Lowry O H Rosebrough N J Farr A L & Randall R J* Protein measurement with the folin phenol reagent J Biol Chem *193* 265–275 1951

Acta path et microbiol scandinav 74 179-188 1968

The Laboratory of Paediatric Pathology (Head J Tygstrup) University Institute of Pathological Anatomy (Head G Teilum) and Division of Haematology (Head S A Killmann) Medical Department A Rigshospitalet (Head K Brøchner Mortensen) Copenhagen Denmark

CHRONIC GRANULOMATOUS DISEASE OF CHILDHOOD

A Morphologic Study

By

PAUL S SYMCHYCH JOHN WANSTRUP and VAGN ANDERSEN

Received 20 iii 68

Chronic granulomatous disease (CGD) is a well defined and fatal syndrome of childhood characterized by inadequate bactericidal function of neutrophil granulocytes When first described the emphasis was placed on the tissue histiocyte and the granuloma as evidenced by titles of the studies by *Landing & Shirkey* (1957) A syndrome of recurrent infection and infiltration of the viscera by pigmented lipid histiocytes and by *Berendes et al* (1957) A fatal granulomatosus of childhood In 1966 *Holmes* and her co workers demonstrated that leucocytes from affected patients could ingest but not digest environmental bacteria With the electron microscope CGD neutrophils have shown persistence of morphologically intact bacteria in the cytoplasm *after* phagocytosis *in vitro* (*Quie et al* 1967) whereas in normal neutrophils the bacteria were seen in all stages of digestion

The purpose of this paper is to describe the histopathological features of three cases of CGD and to re emphasize the value of the combination of pigmented histiocytes and granulomas in the histological diagnosis of this disease

Clinical Features

Until recently CGD was regarded as an X linked recessive disease manifesting itself only in boys However *Baehner & Nathan* (1967 a) described a female patient a product of a first cousin marriage More recently the syndrome has been diagnosed in three negro sisters born to healthy non consanguineous parents (*Azimi et al* 1968) A possible explanation of these conflicting findings is that

This study is supported in part by a grant (to PSS) by the *Charles Percey Foundation* Boston Mass

Patients 1 and 2 were followed in the Paediatric Department (Head P Plum) and in the Children s Allergy Clinic (Head K Wilken Jensen) Rigshospitalet during a large part of their illness We thank the above persons for permission to employ the clinical data of these patients

there may be several genetically determined defects in the bactericidal capacity of neutrophils which may give rise to identical clinical patterns which cannot be separated by current techniques

The hallmark of this disease is the onset in early infancy of repeated infections Typically these are chronic suppurative and granulomatous lymphadenitis draining abscesses parenchymatous granulomas especially of the lung and infected dermatitis often facial and periorificial A wide variety of organisms frequently staphylococci has been cultured from these lesions

No defects of immunoglobulins have been detected and diffuse hypergammaglobulinaemia is often a prominent feature The ability to form circulating antibodies *in response to antigenic stimulation* is unaffected Studies on cellular immunity have likewise failed to disclose any defect Peripheral blood leucocyte counts and morphology are normal, and these patients respond to infection with neutrophil leucocytosis

Phagocytic capacity of neutrophils is normal but their bactericidal capacity is deficient and degranulation and vacuolization after phagocytosis is minimal (*Holmes et al* 1966 *Quie et al* 1967) Thus neutrophils are able to ingest bacteria normally *but cannot destroy them at a normal rate* No defects of lysosomal catabolic enzymes have been demonstrated in CGD neutrophils however after phagocytosis these enzymes are not released into the phagocytic vacuole Likewise leucocytes fail to reduce nitroblue *tetrazolium following phagocytosis and this* property has become the basis of a clinical screening test (*Baehner & Nathan* 1967) The carrier female has been shown to have two populations of leucocytes *approximately equal in size one that functions normally and one that is defective* (*Windhorst et al* 1967 *Andersen et al* 1968)

MATERIAL AND METHODS

The tissue available for study stems from three boys suffering from CGD two brothers whose histories have been presented in detail by *Andersen et al* (1968) and one of the two siblings with CGD studied in another institution (*Jans & Huber* 1967)

Case 1 Boy born in 1949 Recurrent infections began at 3 months of age tonsillitis lymphadenitis of the neck which required surgical drainage on several occasions bronchitis pneumonias gingivitis and fissures in the corners of the mouth At the age of three years and a half he was hospitalized with fever and diarrhea He remained in hospital with septic fevers and anaemia until his death 6 months later The terminal infection was a subphrenic abscess Necropsy (no BS 382/52) was performed and showed hepatic abscesses empyema and pneumonia, and fibrinopurulent pericarditis Pertinent laboratory data include normal granulocyte and lymphocyte counts and leucocytosis during infections

Case 2 The brother of case 1 born in 1959 Infections began at the age of 8 months and included bouts of otitis *media lymphadenitis of the neck requiring* repeated incisions blepharitis panaritia and eczematous lesions about the nose and mouth At the age of 7 years after a symptom free period of nearly 18 months *he was hospitalized for* drainage of cervical lymphadenitis Shortly thereafter he had a subphrenic abscess drained improved and was discharged He developed septic fever and died at home at the age of nearly 8 years Necropsy was not performed but during the course of his illness several surgical biopsies were obtained

Pertinent laboratory data include *normal or elevated granulocyte and lymphocyte* counts and leucocytosis during infections Serum gamma globulins were elevated Reactions of delayed hypersensitivity could be elicited Phagocytic capacity of *neutrophils was examined* on two occasions in 1961 and 1967 and was normal After phagocytosis neutrophils showed morphologically intact ingested staphylococci and no degranulation or vacuolization The nuclei of the phagocytosing granulocytes gradually became pyknotic and fragmented demonstrating the death of the cells

Case 3 A 3 year old boy with a history of recurring otitis and lymphadenitis The diagnosis *of CGD* was confirmed by the inability of the patient s neutrophils

to destroy ingested bacteria (*Jans & Huber* 1967) He died after recurring episodes of high fever Liver tissue from the autopsy was kindly placed at our disposal by Dr *J Huber*

Tissues were fixed in formalin and paraffin embedded in the usual manner In addition to the routine stains sections were stained by the periodic acid Schiff technique Sudan Black Ziehl Neelsen Gram toluidine blue and iron stains Slides were also examined with polarized light and under the fluorescence micro scope

Pathology

The following histopathological description represents a synthesis of all the available material from the three cases

Morphologically the pigmented lipid histiocyte is a cell with a single nucleus and pigmented cytoplasm In unstained preparations and in haematoxylin eosin stained preparations the pigment is yellow brown It is present as a diffuse apparently non granular pigment distributed throughout the cytoplasm This pigment is present in both fresh frozen and formalin fixed paraffin embedded tissue In addition to the diffuse cytoplasmic pigment these cells often have coarse dark brown granules of varying sizes in the cytoplasm These granules have histochemical properties similar to the cytoplasmic pigment The pigmented cell is most readily recognized in slightly diminished light and we have found this useful in examining H & E stained slides

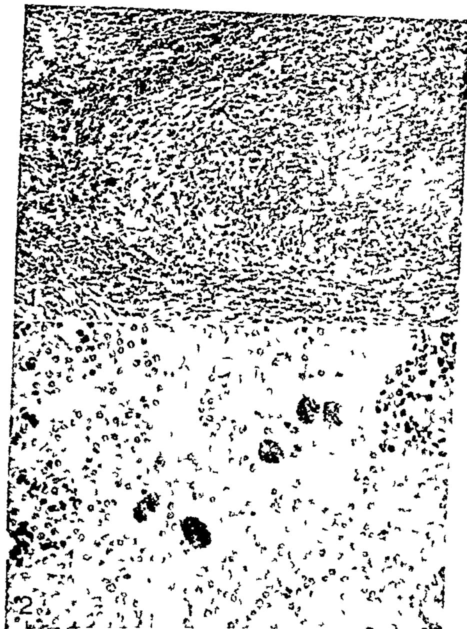
Topographically pigmented histiocytes are found in lymph nodes spleen liver lungs and colon This distribution is in accord with that described by *Carson et al* (1965)

Histochemically the pigment is strongly sudanophilic even in pa raffin sections variably PAS positive and weakly acid fast It is ortho chromatic in toluidine blue and demonstrates autofluorescense No birefringence is noted in polarized light Gram and Ziehl Neelsen stains for organisms are negative The pigment is iron negative

In the *lymph nodes* the characteristic lesions are pigmented histio cytes and caseating granulomas (Figs 1 2 and 5) Pigmented histio cytes are numerous They are located around and between the granulo mas and are also present in areas with marked sinus histiocytosis but with no nearby granuloma Within sinusoids pigmented histiocytes are often seen in clusters of 4 to 8 cells They are occasionally seen in the periphery of lymphoid follicles but not in the germinal centres The pigmented cell is not a constituent of the granuloma itself It should be emphasized that many granulomas have no adjacent pigmented histio cytes

The granulomas are often caseating and bear a striking resemblance to tuberculous granulomas including giant cells of the Langhans type The similarity is such that in many cases including one of ours the initial histological diagnosis is tuberculosis but all stains and cultures for tubercle bacilli are negative Increased numbers of plasma cells are usually found

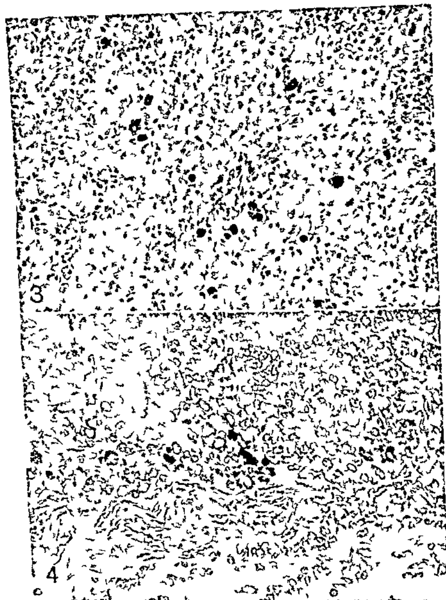
In the *spleen* pigmented histiocytes are found in the sinusoids of the



Figs 1-2

Fig 1 Lymph node case 2 The margin of a characteristic lymph node granuloma This granuloma demonstrates the resemblance to tuberculosis with central necrosis and giant cells Haematoxylin and Eosin × 140

Fig 2 Lymph node case 2 Pigmented lipid histiocytes in the medullary portion of a lymph node Note the large size of the cells and the strong Sudanophilia. Sudan Black × 450

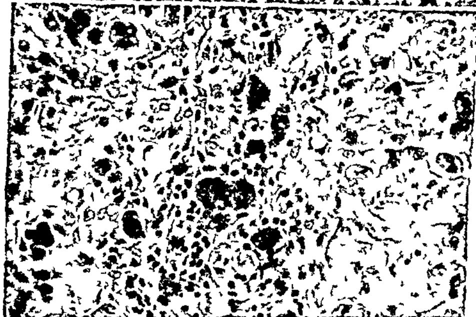


Figs 3 4

Fig 3 Liver case 3 The large deeply staining cells are swollen Kupffer cells containing the pigment characteristic of the disease This photograph illustrates the large numbers of pigmented cells present in most areas of the liver and the ease with which they may be recognized Periodic acid Schiff × 140

Fig 4 Colon case 1 A tangential section to show the lamine propria above the wavy muscularis mucosae and the sub mucosa below Note the darkly staining histiocytes located in the lamina propria Sudan Black × 350

5



6

Figs 5–6

Fig 5 Lymph node case 2 A medullary portion of a node similar to that in Fig 2 illustrating the PAS positive histiocytes Periodic acid Schiff × 450

Fig 6 Liver case 1 A portal area of the liver to show *slight chronic inflammation* and numerous large PAS positive histiocytes Periodic acid Schiff × 350

red pulp in a perivascular distribution and in the perifollicular collars No granulomas are found Plasma cells are increased in number and are often binucleate There seems to be an overall hypocellularity at the expence of the white pulp That is the expected reactive hyperplasia of lympoid follicles is not present

In the *liver* the features are pigmented phagocytic cells chronic inflammation and granulomas (Figs 3 and 6) Nonspecific chronic inflammation is present in the portal areas and granulomas both caseating and non caseating are common Healing by patchy fibrotic scars is prominent Pigmented histiocytes are found in the portal areas and are incorporated into many of the fibrous scars Another striking feature is the presence of pigment morphologically and histochemically indistinguishable from that seen in other histiocytes within a great number of Kupffer cells Such pigment laden Kupffer cells are swollen and at times nearly fill sinusoids

In the *colon* chronic nonspecific inflammation and pigmented histiocytes are the striking features Pigmented histiocytes are present in the lamina propria in large numbers (Fig 4) Scattered pigmented cells are present in the submucosa and as noted in other organs their location is often perivascular Solitary lympoid follicles contain pigmented histiocytes and in this respect resemble the findings in the lymph nodes

The changes in the *lungs* are dominated by severe widespread pneumonia Most of the granulomas are small and range from caseating tuberculoid granulomas to granulomas with varying amounts of intercellular hyaline material The latter which are the most numerous have a resemblance to sarcoidosis because of this peculiar hyalinization Occasional solitary giant cells are found within alveoli and septae A few pigmented histiocytes are seen in the interstitium in a perivascular location None are seen in the granulomas

Skin biopsies show acute and chronic suppurative inflammation with prominent eosinophilia No pigmented histiocytes are noted

In all sections examined a special search was made for the presence of pigmented cells within blood vessels In no instance could we conclusively point to a cell with cytoplasmic pigment similar to that seen in tissues Bone marrow aspirates are also negative in this respect

DISCUSSION

CGD presents certain characteristic features on the basis of which an accurate histological diagnosis may be established As described in our material the following histological criteria may be used

1 The most conspicuous lesion is the *granulomatous tissue reaction often of the tuberculoid type* These appear in a variety of developmental forms but here as in other granulomatous diseases they have no diagnostic specificity per se

2 *Pigmented lipid histiocytes* which may be distributed throughout

the reticuloendothelial system They are found adjacent to but not as a part of granulomas as isolated cells or clusters of cells in non granulomatous areas and often in a perivascular or sinusoidal distribution

3 *Plasmacytic cellular proliferation* the morphological counterpart of the hypergammaglobulinaemia frequently present in these patients

On the basis of these histologic criteria especially the pigmented lipid histiocyte in combination with the granuloma one can confidently suggest the diagnosis of CGD In a large variety of other granulomatous diseases of known and unknown aetiology re examined in this institute we have not encountered such pigment laden cells by routine and special stains

A finding that may have diagnostic importance clinically is the presence of pigmented histiocytes in the gastrointestinal tract In the colon these cells were prominent in the autopsied case and they were found in all of 6 autopsy cases of CGD reported by *Carson et al* (1965) It would therefore seem justified to explore the value of rectal mucosal biopsy as a diagnostic screening adjunct particularly in situations where haematological tests for the disease are not readily available

The nature of the pigment is unknown Initially characterized as a lipochrome there has been little further progress in this direction Our studies generally confirm those of *Landing* (1957) The pigment is insoluble in routine tissue processing strongly sudanophilic variably PAS positive weakly acid fast by the *Ziehl Neelsen* technique and iron negative Intracellular organisms cannot be recognized Whether this pigment represents indigestible bacterial residues or reflects a metabolic aberration with metabolite accumulation in the histiocyte is not known The former possibility brings to mind the PAS positive cytoplasmic bodies present in Whipple s disease which by electron microscopy have been shown to be of bacterial origin—both intact and degenerating organisms (*Rostgaard* 1964) A recent ultrastructural study of splenic histiocytes obtained at autopsy from a patient with CGD did not give any clue as to the origin of the pigment (*Bartman et al* 1967)

Patients with CGD react to ordinary pathogens such as staphylococci by the formation of granulomas In normal individuals these bacteria are not associated with granuloma formation An analogy has been made between patients with CGD and normal patients infected with organisms capable of surviving within mononuclear cells (*Holmes et al* 1966) It is well known that Mycobacteria Brucella and Listeria (all granuloma inducing infective agents) are capable not only of surviving but also of multiplying within phagocytes The parallel is drawn between the intracellular survival of these special micro organisms in macrophages of normal hosts and the survival of ordinary bacteria in neutrophils that are to use *Kahle s* term impotent (1967) However intracellular survival of bacteria is (by itself) not sufficient to explain granuloma formation which also demands the establishment of cellular immunity (*Jensen et al* 193) *Jensen & Bindslev* 1937)

The functional state of the tissue phagocytes in CGD is not known. If the *entire* cellular bactericidal system was deficient it would be expected that these patients would die with their first infection. Furthermore it is established that macrophages are necessary for the full development of immune reactions (*Suter & Ramseier* 1964). Since both humoral and cellular immunity are intact in children with CGD it seems improbable that the macrophages of these patients are as incompetent as the neutrophils. In this connection it may be of importance to note that none of the children suffering from CGD have been reported to have tuberculosis or any of the other diseases associated with the survival of the infecting organisms within tissue macrophages. Moreover the bacteria which infect CGD patients and survive within their neutrophils have not been observed within tissue macrophages. Finally when one compares neutrophils and monocytes from CGD blood during phagocytosis in vitro it is evident that the bacteria thrive and multiply within the neutrophils (*Holmes et al* 1966, *Andersen et al* 1968) but rapidly become smaller, assume a fuzzy outline and are never seen to divide within the monocytes. This indicates that monocytes are probably capable of destroying the ingested bacteria. The indirect evidence cited above suggests to us that CGD is primarily if not entirely a disorder of neutrophil granulocytes and that other phagocytic cells of the body have at least some degree of normal function.

SUMMARY

The pathological findings in three patients with Chronic Granulomatous Disease (CGD) are presented. Special emphasis is placed on the pathognomonic histological combination of pigmented macrophages and granuloma formation. The macrophages which contain a yellow sudanophilic PAS positive pigment are found dispersed in the tissues. Their presence in the submucosa and lamina propria of the large intestine suggests that rectal biopsy may be of help in the diagnosis of CGD.

Unimpaired phagocytosis but defective bactericidal capacity of the neutrophil granulocytes has been demonstrated in this disease. Evidence is presented that the other phagocytic cells of these patients do show at least some degree of normal function.

REFERENCES

Andersen V, Koch C, Vejlsgaard R & Wilken Jensen K: Fatal Granulomatous Disease. Acta Pediat Scand 57 110–114 1968

Ament M P, Brodenbender J G, Hunt R L & Kontras S B: Chronic Granulomatous Disease in Three Sisters. The Lancet 1 208–209 1968

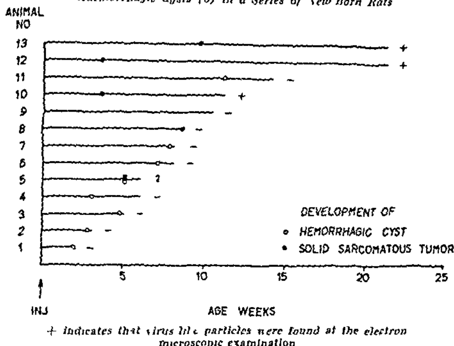
Baehner R L & Nathan D G: Leukocyte Oxidase Defective Activity in Chronic Granulomatous Disease. Science *155* 835–836 1967

microscope the final pellet was suspended in saline and (sometimes divided in aliquots of 2-3 ml) injected i m into three week old chickens strain White Leghorn In another series of newborn rats injected with RSV as above the injection site was excised on the first day and on every second day after the injection until the time when tumours appeared in other rats of the same series The specimen from the injection site was divided in two parts one was processed for examination in the electron microscope the other homogenized and injected into chickens

In Table 1 the records of the rats with cysts and sarcomas are presented

TABLE 1

Injection of Rous Virus (SR) and Development of Solid Tumours (●) and Haemorrhagic Cysts (o) in a Series of New Born Rats



+ *indicates that virus like particles were found at the electron microscopic examination*

Electron microscopy Tissue specimens were removed from the animals at various intervals before and after tumour development Special care was taken to obtain representative tissue from all parts of the tumour Tissue blocks about 1 mm³ were cut and fixed as soon as possible in 3 per cent glutaraldehyde saline (pH adjusted to 7 2 with a few drops of phosphate buffered saline) for between 4 24 hours After washing in buffered saline the specimens were postfixed in 3.33 per cent OsO_4 s collidine (Wood & *Luft* 1965) solution for 60 min at +4 C Repeated washing was followed by dehydration in graded ethanol The embedding was performed in Epon 812 with propylen oxide as an intermediate solvent (*Luft* 1961) Some of the specimens were embedded in Westopal (according to *Ryter* & *Kellenberg* 1958) Sectioning was performed on a LKB Ultrotome and the specimen grids were examined in a Zeiss EM 9 electron microscope

RESULTS

The ultrastructural appearance of Rous rat sarcoma is shown in Fig 1 As in the light microscope two different types of cells can be distinguished

One is elongated having a large "blown up nucleus with a finely

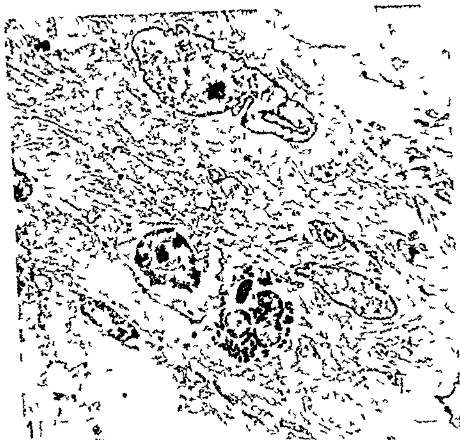


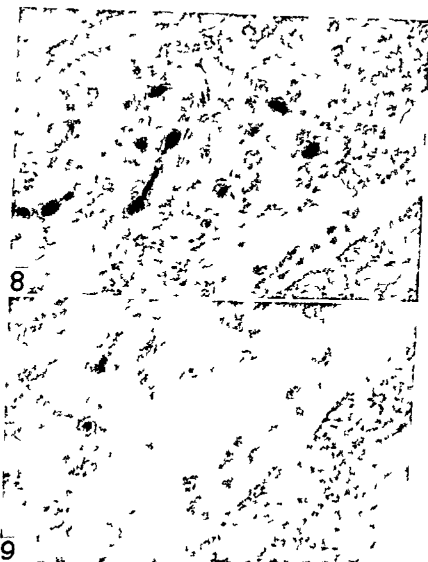
Fig 1

Partly necrotic tumour from animal No 12 macroscopically evident for over 4 months before specimen was examined In the upper field is seen a sarcoma cell with blown up nucleus Just below and to the left of the centre the other type of cell is seen A granulocyte is seen as comparison × 3 600

granular appearance and as a rule a small centrally located nucleolus The nuclear membrane is usually smooth but sometimes indentations or other irregularities are seen The cytoplasm contains a well developed rough endoplasmatic reticulum A few rounded mitochondriae are found In general the appearance of this cell is closely related to that of rat fibroblasts and as can be seen in the figure bundles of collagen interlace between the cells

The other cell is generally smaller more irregularly outlined and often shows irregularities or infoldings of the nuclear membrane with heavy condensation of the nuclear chromatin along the membrane The cytoplasm shows a few scattered cell organelles mostly primitive mitochondriae Occasionally "dense bodies are found

Virus particles were seldom found on electron microscopic analysis of excised tissue from the injection site In Fig 2 is demonstrated an



Figs 8–9

Fig 8 Survey picture of a part of the cytoplasm of a tumour cell containing various cell organelles of unknown origin This unusual field demonstrates a possible source of false virus like particles Animal No 1? × 37 500

Fig 9 High magnification of one of the virus like particles in Fig 8 Particle size 75 mμ × 67 500

of the abdominal aorta A few axillary cysts were found but were not examined So far no virus like particles were seen in the cyst walls

Rat No 13 showed a pea sized metastasis at the mesenterial attachment of the small intestine No virus like particles were found in the metastasis

In

transferred Rous rat sarcomas were studied in the electron microscope In none of them were virus like particles observed

It must be stressed that the number of particles was very low in all the sarcomas In one of the tumours (animal No 8) no particles were observed and in another tumour (No 5) the particles observed could not be classified (indicated by a question mark in Table 1)

Homogenates from the RR sarcomas injected into chickens did not elicit any tumours nor could infectious virus be found in the tissue from the injection site from the third day onwards The chickens were observed for about 6 months Excised homogenized tissue from the injection site the day after the injection of RSV subsequently induced sarcomas in chickens

DISCUSSION

By the electron microscope virus like particles have been found in many experimental and other tumours Critical interpretation of the findings has pointed out many difficulties and pitfalls *i e* passenger viruses secretory granules vesiculated structures containing dense material or tangentially sectioned cell organelles (*Bernhard* 1958 1960 *Gross* 1961 *Smith et al* 1964 *Benyesh Melnick et al* 1964 *Dmochowski et al* 1964 *Anderson* 1965 *Arnoult & Haguenau* 1966)

The morphology of the Rous virus particles is well known (*Bernhard et al* 1956 *Epstein* 1956 and 1958 *Haguenau et al* 1960 and 1962 *Dourmashkin & Simons* 1961) The RSV belongs to the avian leucosis sarcoma complex showing a double or triple layered outer membrane surrounding a dense nucleoid of RNA The RSV particles have a diameter of about 75 mμ (*Bernhard et al* 1956) or rather less (*Gaylord* 1955) while others (*Haguenau & Beard* 1962) have found 70–80 mμ

The particles observed in this investigation were akin to virus of the avian leucosis group in their morphology but were generally found to have a diameter slightly above the values quoted (from 80 mμ to 110 mμ)

The fact that only very few particles were found is not remarkable as they are rarely seen even in the Rous sarcoma in chicken One virus producing cell per 50 tumour cells in high producing sarcomas to one virus releasing cell per 3000 in the low producing tumours (*Epstein* 1956 1958) and only a few virus particles per section from these cells

It seems unlikely that the observed particles represent an indigenous virus already present in the rats They occurred at the site of the injected virus suspension and in the primary solid sarcomas only not in the cyst walls or in the serially transplanted tumours It is more difficult to exclude the possibility that the particles are impurities in the injected material It seems likely that some of the structures of unknown origin demonstrated in Fig 8 represent phagocytized impurities Their non virus nature could be revealed by examination of serial sections

and they could clearly be distinguished from the observed structures having the morphological character of virus of the avian leucosis group

If it is assumed that the particles observed represent Rous virus they might either have been synthetisized by the sarcoma cells or been transported into the cell via phagocytosis or pinocytosis from the injected virus suspension. The fact that the particles were found intracellularly is consistent with phagocytotic activity. It is difficult, however, to understand how phagocytized virus particles can remain seemingly unaffected for several months undestroyed by lysosomal enzymes in the cells. It might be that the outer envelopes of the particles render them resistant to intracellular destructive forces for a considerable time.

Intracellularily located virus particles are well known in the murine leukaemia and milk agent systems. Type A according to *Bernhard & Guérin (1958)* and *Bernhard (1960)* or the sub group A as proposed by *de Harven* (1962) is thought to remain intracellularily for a long time but show the characteristic doughnut appearance and do not achieve the dense central nucleoid until released extracellularily, i.e. as type C particles. It has been shown that mouse leukaemia virus can induce leukaemia in rat: in the electron microscope both type A and C particles as well as budding phenomena are found (*Dmochowski et al* 1962 and 1964). The morphology and size of the C particles are comparable with the particles found in the present investigation and the possibility that they represent passenger virus cannot be excluded. Synthesis of virus particles has been observed in meningeal sarcomas in dogs induced by intracranial injection of RSV (*Rabotti et al* 1966, *Bucciarelli et al* 1967). The particles were seen budding from cell membranes mostly from the outer membrane but also into intracellular vesicles. The occurrence of virus particles in Rous sarcoma in dogs favours the hypothesis that the particles observed are synthetisized by the sarcoma cells. However, in no section so far examined has there been any sign of virus formation at the cell membranes (or in the neighbourhood of the Golgi complex) as observed by *Bucciarelli et al* (1967) in the meningeal tumours.

Biological testing of the virulence of the virus particles observed in the meningeal sarcoma gave negative results as well as the biological assay in the present work. The failure to demonstrate infectivity might be explained by the low number of virus particles found in the rat sarcoma cells.

The observation that virus particles were found only in one of the two types of cells in the Rous rat sarcoma which can be identified in the electron microscope warrants further investigations. Two types of cells are also seen in Rous sarcomas in chicken. It is not known, however, whether both of these synthesize infectious virus.

The absence of virus like particles in the cyst walls confirms the observation made by others that infectious virus is not present in the

cysts (*Svoboda & Grozdanović* 1959) As in the solid sarcoma however the viral genome is retained in the cyst walls (*Svet Moldavsky & Scorikova* 1960 *Munroe & Southam* 1964)

SUMMARY

Neoplastic lesions (solid sarcomas haemorrhagic cysts) induced in rats by Rous sarcoma virus (strain SR) were studied in the electron microscope In 3 out of 5 rat sarcomas sparse virus like particles were seen They were localized intracellularly and their morphology were akin to that of the avian sarcoma leucosis group At the site of the injected RSV similar particles were observed In the haemorrhagic cysts or in serially transferred sarcomas no particles were found The nature of the virus like particles is discussed

REFERENCES

1 *Ahlstrom C G* Neoplasms in mammals induced by Rous chicken sarcoma material NCI Monograph *17* 299–319 1964

2 *Andersson D R* Subcellular particles associated with human leukemia as seen with the electron microscope In Methodological approaches to the study of leukemias The Wistar Inst Symposium Monograph (ed Defendi V) *II* 4 1965

3 *Arnoult J & Haguenau F* Problems raised by search for virus particles in human leukemia A study with the electron microscope of blood plasma cerebrospinal fluid and megakaryocytes from bone marrow J Nat Canc Inst *36* 1089 1109 1966

4 *Bengesh Melnick M Smith K O & Fernbach D J* Studies on human leukemia III Electron microscopic findings in children with acute leukemia and in children with infectious mononucleosis J Nat Canc Inst 33 571–580 1964

5 *Bernhard W* Electron microscopy of tumor cells and viruses A review Cancer Res *18* 491–509 1958

6 *Bernhard W* The detection and study of tumor viruses with the electron microscope Cancer Res *20* 712–727 1960

7 *Bernhard W & Guérin M* Présence de particules d aspect virusal dans les tissus tumoraux de souris atteintes de leucémie spontanee Compt Rend Acad Sci 247 1802–1805 1958

8 *Bernhard W Oberling C & Vigier P* L ultrastructure de virus dans le sarcome de Rous leur rapport avec le cytoplasme de cellules tumorales Bull Cancer 43 407–422 1956

9 *Bucciarelli E Rabotti G F & Dalton A J* Ultrastructure of meningeal tumors induced in dogs with Rous sarcoma virus J Nat Canc Inst 38 359–381 1967

10 *Dmochowski L Grey C F Padgett F Langford P L & Burmester B R* Submicroscopic morphology of avian neoplasms VI Comparative studies on Rous sarcoma visceral lymphomatosis erythroblastosis myeloblastosis and nephroblastoma Tex Rep Biol Med 22 20–60 1964

11 *Dmochowski L Gross L & Padgett F* Electron microscopic studies of rat leukemia induced with mouse leukemia virus Proc Soc Exp Biol Med 110 584–588 1962

12 *Dmochowski L Padgett F & Gross L* An electron microscope study of rat leukemia induced with mouse leukemia virus (Gross) Cancer Res 24 869 899 1964

13 *Dourmashkin R R & Simons P J* The ultrastructure of Rous sarcoma virus J Ultrastruct Res 5 50–522 1961

14 *Epstein M A* The identification of the Rous virus A morphological and biological study Brit J Cancer *10* 33–48 1956

15 *Epstein M A* Observations on the Rous virus purification and identification of the particles from solid tumours Brit J Cancer *12* 248–255 1958
16 *Febvre H Rotschild L Arnoult J & Haguenau F* In vitro malignant conversion of rat embryonic cell lines with the Bryan strain of Rous sarcoma virus NCI Monograph *17* 459–477 1964
17 *Gaylord W H Jr* Virus like particles associated with Rous sarcoma as seen in sections of the tumor Cancer Res *15* 80–83 1955
18 *Gross L* Oncogenic viruses p 252 Pergamon press (New York Oxford London & Paris) 1961
19 *Haguenau F & Beard J W* The avian sarcoma leukosis complex its biology and ultrastructure In Ultrastructure in biological systems Vol *1* pp 1–60 Acad Press (New York & London) 1962
20 *Haguenau F Febvre H & Arnoult J* Ultrastructure du virus de sarcome de Rous cultivé in vitro Compt Rend Acad Sci 250 1747–1749 1960
21 *Haguenau F Febvre H & Arnoult J* Mode de formation intracellulaire du virus de sarcome de Rous J Microscopie *1* 445–454 1962
22 *de Harven E* Ultrastructural studies on three different types of mouse leukemia In Ultrastructure in biological systems Vol *1* pp 183–206 Acad Press (New York & London) 1962
23 *Klement V & Vesely P* Tumour induction with Rous sarcoma virus in hamsters and production of infections Rous sarcoma virus in an heterologous host Neoplasma *12* 147–153 1965
24 *Luft J H* Improvements in epoxy resin embedding methods J Biophys Biochem Cytol *9* 409–414 1961
25 *Moloney J B* Biological studies on a lymphoid leukemia virus extracted from sarcoma 37 I Origin and introductory investigations J Nat Canc Inst *24* 933–951 1960
26 *Munroe J S Shipkey F Erlandsson R A & Windle W F* Tumors induced in juvenile and adult primates by chicken sarcoma virus NCI Monograph *17* 365–390 1964
27 *Munroe J S & Southam C M* Oncogenicity of two strains of chicken sarcoma virus for rats J Nat Canc Inst 32 591–623 1964
28 *Rabotti G F Bucciarelli E & Dalton A J* Presence of particles with the morphology of viruses of the avian leukosis complex in meningeal tumors induced in dogs by Rous sarcoma virus Virology 29 684–686 1966
29 *Ryter A & Kellenberger E* L inclusion au polyester pour l ultramicrotomie J Ultrastruct Res 2 200–214 1958
30 *Smith K O Benyesh Melnick M & Fernbach D J* Studies on human leukemia II Structure and quantitation of myxo virus like particles associated with human leukemia J Nat Canc Inst 33 557–570 1964
31 *Svet Moldavsky G J* Pathogenicity of Rous sarcoma virus for mammal Sarcomas in rats further studies on cyst haemorrhagic disease and an attempt at isolating infectious ribonucleic acid from Rous sarcoma Acta Virol *5* 167–177 1961
32 *Svet Moldavsky G J & Skorikova A S* The pathogenicity of Rous sarcoma virus for mammals Detection of virus and of antigenic substances of Rous sarcoma in the cyst haemorrhagic disease of albino rats Acta Virol *4* 47–51 1960
33 *Svoboda J* Presence of chicken tumour virus in the sarcoma of the adult rat inoculated after birth with Rous sarcoma tissue Nature (London) *186* 980 981 1960
34 *Svoboda J Chyle P Simkovic D & Hilgert I* Demonstration of the absence of infectious Rous virus in rat tumour XC whose structurally intact cells produce Rous sarcoma when transferred to chicks Folia Biol (Praha) *9* 77–81 1963
35 *Svoboda J & Grozdanovič J* Heterotransplantation of Rous sarcoma in young rats Folia Biol *5* 46–50 1959
36 *Temin H M & Rubin H* Characteristics of an assay for Rous sarcoma virus and Rous sarcoma cells in tissue culture Virology *6* 669 688 1958
37 *Wood R L & Luft J H* The influence of buffer systems on fixation with osmium tetroxide J Ultrastruct Res *12* 22 45 1965
38 *Zilber L A* Pathogenicity and oncogenicity of Rous sarcoma virus for mammals Progr Exp Tumor Res 7 1 48 1965 (Karger Basel/New York)

Acta path et microbiol scandinav 74 199–204 1968

The Blood Bank (Chief Dr G Munk Andersen) and the Department of Neurology (Chief Dr H Haugsted) The City and County Hospital Odense Denmark

IMMUNOFLUORESCENT TECNIQUE USED IN THE STUDY OF SERUM FROM PATIENTS WITH MULTIPLE SCLEROSIS, FOR ANTIBODIES TO PAPAINIZED BRAIN TISSUE

By

HANS DIEDERICHSEN and INGEBORG COSMUS PINDT

Received 4 ii 68

Since 1934 when *Sachs & Steiner* (7) demonstrated the presence of complement fixing antibodies to brain tissue extract in the sera of patients with multiple sclerosis many attempts have been made to demonstrate antibodies to brain tissue in patients suffering from this disease but with very varying results Applying the complement fixation technique the frequency of positive results varies from 83 3 per cent (5) to 0 per cent (4) of the cases investigated

Ross (6) using a trypsin extract of homogenized brain tissue as an antigen found positive reactions in 40 per cent of patients with multiple sclerosis compared with 2 per cent in normal individuals by means of the Ouchterlony gel diffusion technique By indirect immunofluorescent staining *Allerand & Yahr* (1) found fluorescence of glial cells and myelin sheaths after incubation with sera from patients with multiple sclerosis and other neurological disorders as well as from normal individuals

The results obtained by *Ross* (6) might indicate the presence of antibodies to enzymatically degraded brain tissue in patients with multiple sclerosis The purpose of the present study is to employ the indirect immunofluorescent technique of *Coons* (2) in the investigation of circulating antibodies to brain tissue sections which have been exposed for a brief period to the proteolytic enzyme papain and to compare the results obtained when non papainized sections were studied

MATERIAL

Antigen Cortex and subcortical white matter from a normal parieto occipital lobe removed during operation frozen in liquid oxygen less than three hours after operation and stored at —40 C until use

Proteolytic enzyme Papain Merck 1 000 units/g Employed in a concentration of 0 1 per cent in 0 9 per cent NaCl During preliminary experiments higher concentrations were found to cause pronounced destruction of the tissue

Fluorescein-conjugated antihuman globulin Lyophilized commercial preparation from Merieux 1 ml of the dissolved preparation was absorbed for thirty minutes at room temperature by lyophilized human brain in order to reduce non specific fluorescence

Phosphate buffered saline 0 15 M NaCl and 0 01 M phosphate buffer pH 7 2

Phosphate buffered glycerine saline Equal volumes of glycerine and phosphate buffered saline pH 7 2

METHOD

Tissue sections 5 microns thick were cut in a cryostate at —20 C, transferred to glass slides and air dried for at least thirty minutes at room temperature All sections were used the same day

The mounted sections were immersed for one minute in a 0 1 per cent solution of papain and then washed four times over periods of one minute in phosphate buffered saline After being papainized the sections were first incubated in a moist chamber with one drop of serum for thirty minutes at room temperature They were then washed in phosphate buffered saline for three periods of five minutes each After washing the sections were incubated with one drop of fluorescein conjugated antihuman globulin in a moist chamber for thirty minutes at room temperature Then the sections were washed again with phosphate buffered saline for three periods of five minutes each The surface of the glass slides around the sections was dried thoroughly after each washing The sections were covered with phosphate buffered glycerine saline and cover slides

Sections not treated with papain and not washed were incubated in the same way with serum and fluorescein conjugated antihuman globulin

Reading was carried out employing a Zeiss fluorescence microscope with an Osram HBO mercury lamp exciter filter III and barrier filter 44 nm Dark field condenser was applied For photographs Ektachrome film was used Exposure time fifteen seconds

We studied sera from twenty five patients with multiple sclerosis in all phases of the disease sera from eight patients with cholecystitis and sera from thirty healthy blood donors All sera were stored at —40 C before being used

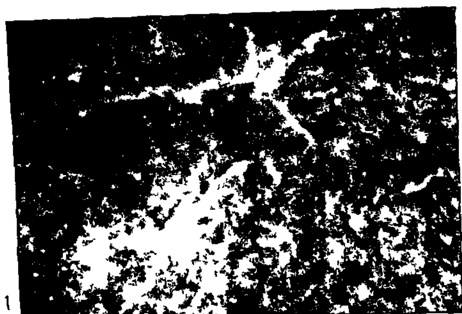
All sera were studied in the undiluted stage and diluted 1 10 with both papainized and non papainized brain tissues

RESULTS

In non papainized unfixed sections fluorescence of scattered fibrous astrocytes in the white matter was found with undiluted sera from patients with multiple sclerosis and cholecystitis and from healthy donors (Fig 1) The staining was relatively weak and not all astrocytes in the section seemed to stain The fluorescence was localized to the cytoplasm and the glial processes The cell nucleus did not stain The intensity of the fluorescence observed in patients with multiple sclerosis and in other subjects did not differ The myelin sheaths did not stain In the cortex neither glial cells nor neurons stained In all sera the fluorescence of the astrocytes was very weak or completely absent with sera diluted 1 10 The reactions with sera from patients with multiple sclerosis were weakened to the same extent as others at this degree of dilution

The application of either serum or fluorescein conjugated antihuman globulin produced no astrocyte staining (Fig 2) whereas fluorescein conjugated antihuman globulin resulted in fluorescence of the vessels of the cortex

1



2



F g 1

Staining of fibrous astrocytes after applicati)n of serum and fluoresceinconjugat d antihuman gl bulin In relation to the a troc)tes are se n)ellow autofluorescent granules Original magnification × 490

Fig 2

No taining f fib ous astrocyte after application of flu resceinconjugate l anti hum n globulin There are yellow autofluorescent granules Original magnification × 480

Also in papainized tissue the astrocytes in the white matter stained but the intensity was relatively weak as compared with non papainized tissue Apart from this no difference was observed between papainized and non papainized tissue

In sections dried overnight at room temperature and thereafter fixed in 95 per cent ethanol for thirty seconds no astrocyte staining was observed or any other specific fluorescence with five sera from patients with multiple sclerosis and *five sera from healthy donors* But a non specific staining of vessels occurred resembling that occurring after application of fluorescein conjugated antihuman globulin alone

In order to confirm that the astrocyte staining was caused by a direct binding of a serum factor to the astrocytes five sera from patients *with multiple sclerosis and five sera from healthy donors* were absorbed *with brain tissue before being applied to non fixed non papainized* sections The sera were absorbed twice 30 mg of homogenized lyophilized normal brain tissue being added each time to 0 6 ml of serum Following frequent shaking at room temperature for thirty minutes at both additions, the suspension was centrifuged and the supernatant applied to the brain sections Incubation washing and application of fluorescein conjugated antihuman globulin was performed according to the method described above None of the absorbed sera produced staining of the astrocytes

Sera from patients with systemic lupus erythematosus which sera are known to react with cell nuclei were also studied both with papainized and non papainized sections However the finding differed so greatly from the above and also from the results expected that they will be reported on later

DISCUSSION

One of the modes of provoking autoantibody *formation is supposed to* be the unmasking of usually hidden determinant groups in the tissue *(3) Since these groups are foreign to the mechanism underlying the* formation of antibodies they might cause production of antibodies to own tissues Such an unmasking might occur when the tissue is treated with proteolytic enzyme The results obtained by *Ross (6) might* support the assumption that this could happen in multiple sclerosis since the antibodies demonstrated by him in this disease were directed against enzymatically degraded tissue

In the present study the attempts to demonstrate the presence of antibodies to papainized brain tissue using the *indirect method of* Coons produced negative results Insofar as antibodies to proteolyzed brain tissue are present in patients with multiple sclerosis it seems likely that a possible cause of the negative result of the present study might be that part of the degraded tissue which acts as an antigen in multiple sclerosis was *deliberated* and washed away during the enzyme

treatment and the subsequent washing. Indeed *Ross* (6) demonstrated the presence of antibodies to the dissolved proteins, after treatment of brain tissue with trypsin.

Sera from patients with multiple sclerosis and cholecystitis as well as sera from healthy donors showed fluorescence of astrocytes in non-fixed sections. It seems likely therefore that the reaction is caused by a non-specific binding of protein to the astrocytes. Probably the astrocyte staining is decided by the same serum factor which both in patients with multiple sclerosis and in normal individuals reacts with formalinized tannin treated sheep blood cells coated with potassium chloride extract of homogenized normal brain tissue (unpublished study by the present authors). *Yahr & Allerand* (8) suggested that the serum factor reacting with astrocytes might be present in higher concentrations in patients with diseases such as multiple sclerosis than in others. The present study does not confirm this assumption since all reactions both in patients with multiple sclerosis, healthy donors and patients with cholecystitis became negative or much weaker when serum diluted to 1:10 was used.

Contrary to *Allerand & Yahr* (1) the present investigation revealed staining of the astrocytes only and no staining of the myelin sheaths. Allerand & Yahr used fixed sections which in the present study were found to be incapable of reacting with serum. We cannot explain the difference between our findings and those obtained by Allerand & Yahr.

SUMMARY

Twenty five sera from patients with multiple sclerosis in all phases of the disease, eight sera from patients with cholecystitis and thirty sera from healthy donors were studied by means of the indirect method of Coons for the presence of antibodies to papainized and non-papainized brain tissue.

Specific antibodies were not found in the patients with multiple sclerosis neither to papainized nor to non-papainized brain tissue. All sera showed non-specific reactions with fibrous astrocytes in the white matter. These reactions either disappeared or became much weaker when the serum was diluted to 1:10 both in healthy donors, in patients with cholecystitis and in those with multiple sclerosis. None of the sera studied reacted with astrocytes following absorption with lyophilized brain tissue.

REFERENCES

1. *Allerand C D & Yahr M D*: Gamma globulin affinity for normal human tissue of the central nervous system. Science *144*: 1141–1142, 1964.
2. *Coons A H & Kaplan M H*: Localization of antigen in tissue cells. J Exp Med *91*: 1–13, 1950.
3. *Holborow E J*: An ABC of modern immunology VIII.—Defects and evasions. Lancet *I*: 1209–1210, 1967.

4 *MacLeod I Ridley A R Smith C & Field E J* Failure to demonstrate circulating antibody to alcoholic brain extracts in multiple sclerosis Brit med J *1* 1575-1578 1962
5 *Raskin N* Antibrain antibodies in multiple sclerosis Arch Neurol Psychiat 73 645-655 1955
6 *Ross J* Über Nachweis und Prufung der Spezifitat von prazipitierenden Hirnantikorpern im Serum von Kranken mit Multipler Sklerose und disseminiertem Lupus erythematodes Zeitschr Immun Allerg *126* 59-64 1964
7 *Sachs H & Steiner G* Serologische Untersuchungen bei Multipler Sklerose Klin Wschr *II* 1714-1717 1934
8 *Yahr M D & Allerand C D* Brief report Affinity of gamma globulin for human glia and myelin P 204-206 In Remote effects of cancer on the central nervous system Ed Lord Brain & Forbes Norris Jr Grune & Stratton New York 1965

Acta path et microbiol scandinav 74 205-213 1968

The Cytology Laboratory Department of Pathology Karolinska Institute
Sabbatsberg Hospital Stockholm Sweden

SPUTUM CYTOLOGIC CHANGES IN SMOKERS AND NON SMOKERS IN RELATION TO CHRONIC INFLAMMATORY LUNG DISEASES

By

MAGNUS NASIELL

Received 2 iv 68

The statistical association of smoking, particularly of cigarette smoking and lung cancer is generally accepted Histopathological studies of the bronchial mucosa of smokers have shown a connection between smoking and certain alterations in the epithelium specially metaplasia and cellular atypia Such changes are considered to represent a premalignant stage and often occur diffusely in the bronchial mucosa in established lung cancer (1 6 7 12 14 15 20) It has also been suggested in a sputum cytologic study that such changes are more common in smokers than in non smokers (19)

The existence of a statistical association between smoking and chronic inflammatory lung diseases (11 21) and also between such diseases and lung cancer has been indicated (3 4 5 8 9 22) Histopathological studies of the frequency of metaplasia and atypia of the respiratory mucosa in well defined chronic inflammatory lung diseases are comparatively few (2 18) In one study it was pointed out however that changes indicating epithelial injury were much more common in patients with active pulmonary tuberculosis and bronchiectasis than in a control material (18) Sputumcytologic studies of these conditions are few (10 13 18) Such a cytologic investigation using partly the same material as that employed in the present study showed a substantially higher incidence of the phenomenon ACCF (abnormal columnar cell findings indicating epithelial injury) and metaplasia in cases with active pulmonary tuberculosis than in a control material (18) But in the former investigation the patients smoking habits were not taken into account when the groups were compared

The purpose of the present study was to compare by sputum cytologic technique the effect of cigarette smoking on the respiratory mucosa in subjects without lung affections and in patients with chronic inflammatory lung diseases It was considered to be of special interest to ascertain how the epithelial picture is influenced by both factors

4 *MacLeod I Ridley A R Smith C & Field F J* Failure to demonstrate circulating antibody to alcoholic brain extracts in multiple sclerosis Brit med J *I* 1525–1528 1962

5 *Raskin N* Antibrain antibodies in multiple sclerosis Arch Neurol Psychiat *73* 645–655 1955

6 *Ross J* Über Nachweis und Prufung der Spezifität von prazipitierenden Hirnantikorpern im Serum von Kranken mit Multipler Sklerose und disseminiertem Lupus erythematodes Zeitschr Immun Allerg 126 59–64 1964

7 *Sachs H & Steiner G* Serologische Untersuchungen bei Multipler Sklerose Klin Wschr *II* 1714–1717 1934

8 *Yahr M D & Allerand C D* Brief report Affinity of gamma globulin for human glia and myelin P 204–206 In Remote effects of cancer on the central nervous system Ed Lord Brain & Forbes Norris Jr Grune & Stratton New York 1965

TABLE 1

Number Mean Age Sex Distribution Smoking Habits and other Characteristics in the Examined Groups

	Total No Cases	Mean Age Years	Males %	Town Residents	Characteristics
Without Lung Diseases					
Non Smokers	50	57 1	49	All	No previous chronic lung disease Never smoked regularly
Smokers	398	45 6	73	All	No previous chronic lung disease More than 9 cigarettes/day (72% smoked ≥ 20 cigarettes/d)
With Chronic Inflam Lung Diseases					
Non Smokers	123	60 0	61	≈2/3	69 patients with active pulm tuberculosis 54 patients with chronic bronchitis or bronchiectasis Never smoked regularly
Smokers	147	53 8	89	≈2/3	104 patients with active pulm tuberculosis 43 patients with chronic bronchitis or bronchiectasis More than 9 cigarettes/day (15 % smoked ≥ 20 cigarettes/day)
Squamous Cell Undiff Bronchogenic Carcinoma (Smokers)	101	63 0	97		65 patients with squamous cell carcinoma 36 patients with undifferentiated carcinoma (More than 9 cigarettes/day)

Table 1 shows the number age and sex distribution smoking habits and other characteristics of the subjects in the different groups comprised in the material

The cytologic material consisted of sputum samples including not less than one adequate specimen usually including four smears for each case Samples from the non smokers without lung affections were largely obtained by aerosol produced sputum A superheated mixture of propyleneglycol and saline was applied for this purpose The Papanicolaou staining method was used

All the series in the investigation were examined twice Cases in which the second screening revealed findings other than those observed at the first were further analysed and if possible more material was examined All the specimens were examined by specially trained cytotechnologists with considerable experience of pulmonary cytology All the specimens with positive findings together with numerous control samples from the negative cases have been reviewed by the author It was only by way of exception that the final diagnosis and the histological diagnosis respectively were known at the primary cytologic examination No series showed a difference in the frequency of any alteration greater than 3 5 per cent when the primary and the final results were compared

In order to evaluate the frequency figures for the series compared two by two contingency table test at 05 significance level and one degree of freedom ($\chi^2 = 3.84$) and the standardized population method were used The statistical methods were described in greater detail in a previous paper (1B)

CYTOPATHOLOGIC FINDINGS IN PREVIOUS STUDIES

The cytologic epithelial changes i e ACCF metaplasia and atypical metaplasia investigated in this study have been described in detail in earlier reports (16 17) Consequently only a few brief comments are added here Abnormal columnar cell findings (ACCF) imply the occurrence of numerous degenerated columnar cells in cytologic lung specimens (17) The histological background to ACCF seems to be a cleavage of the bronchial mucosa and exfoliation of degenerated columnar cells (15 17) These columnar cell changes were shown to be correlated with metaplasia and bronchogenic carcinoma (17) It is assumed that these alterations may be a starting point for the metaplastic process (15 17)

Metaplasia includes both the non atypical and the atypical form Metaplastic cells in lung cytology specimens have a characteristic appearance which differs distinctly from that of oral squamous cells and respiratory columnar cells It is not possible to differentiate, cytologically between squamous cell metaplasia and transitional cell metaplasia The occurrence of metaplastic cells has also been shown to be significantly related to bronchogenic carcinoma (17 18) Atypical (dyscaryotic) or dysplastic cell alterations are almost exclusively found in metaplastically changed epithelium (15) Such lesions are regarded as important phenomena during the neoplastic process in the bronchi The atypical metaplastic changes in the bronchial epithelium resemble morphologically the pre malignant (dysplastic) alterations in the uterine cervix

Cases with only isolated metaplastic cells displaying nuclear atypia have not been recorded as atypical metaplasia Hence, all the cases which here are referred to this group exhibit a distinctly atypical cell picture with moderate or pronounced nuclear atypia With regard to such abnormal cells it should be pointed out that nothing but cells which morphologically are definitely non malignant have been designated as atypical metaplastic cells

RESULTS

The incidence of the cytological changes in the different groups is shown in Table 2 As there was a certain degree of variability in the age and sex distribution in the different groups included in the material it was investigated by the standardized population method whether this variability could explain the differences in the frequency of the cytological changes This however was not found to be the case In the following report χ values are inserted only when there was a significant difference between two groups

It is evident that the incidence of all three cytological phenomena examined was significantly higher in the smokers than in the non smokers in the material without lung diseases as well as in the material with chronic *inflammatory affections*[1]

Also the group of non smokers with chronic inflammatory lung lesions exhibited a frequency of all three phenomena which was significantly higher than the frequency observed among non smokers without lung disorders

A comparison of the smokers in the two groups shows only a slight predominance of ACCF and metaplasia in the group of chronic inflam

---

[1] Without lung diseases $\chi^2 = 16\ 33$ and 11 for ACCF metaplasia and atypical metaplasia respectively With lung diseases $\chi^2 = 8\ 2\ 15$ and 13 for ACCF meta plasia and atypical metaplasia respectively

plasia and atypical metaplasia respectively

[2] $\chi = 4\ 8\ 8\ 7$ and 7 5 for ACCF metaplasia and atypical metaplasia respectively

TABLE 2

Incidence of Cytological Changes

	Total No Cases	Cases with ACCF		Cases with Metaplasia		Cases with Atyp Metaplasia	
		No	(%)	No	(%)	No	(%)
Without Lung Disease							
Non Smokers	50	8	(16)	9	(18)	2	(4)
Smokers	398	185	(46)	247	(62)	107	(27)
With Chronic Inflam Lung Diseases							
Non Smokers	123	42	(34)	53	(43)	28	(23)
Smokers	147	77	(52)	99	(67)	65	(44)
Squamous Cell Undiff Bronchogenic Carcinoma (Smokers)	101	57	(56)	79	(78)	49	(49)

Including non atypical and atypical metaplasia (dysplasia)

mation the differences are not significant Atypical metaplasia exhibits a significant predominance however in the group with chronic inflammation ($\chi^2 = 14$)

It is also evident that the incidence figures for all the three alterations are higher in smokers without lung diseases than in non smokers with chronic infectious lung disease The difference in frequency is notable with regard to ACCF and metaplasia

Finally if the groups of smokers without lung diseases and those with chronic inflammatory affections are compared with the lung cancer series the figures for ACCF and metaplasia will be seen to increase in the sequence no lung disorders—chronic inflammation—lung cancer The differences are slight and not statistically significant with one exception the carcinoma series exhibits a significantly higher rate of metaplasia than the series of smokers without pulmonary diseases ($\chi^2 = 8.6$) With regard to atypical metaplasia there is as mentioned above a significant difference between the groups without lung affections and those with chronic inflammation In this respect the latter group showed however a figure only slightly lower than that for the cancer series This difference was not significant

In the group of chronic infectious diseases it was also checked whether the patients with active pulmonary tuberculosis differed from those with non tuberculous lung affections as regards the various cytologic parameters The difference never exceeded 2 per cent in the non smokers Among the smokers there were certain disparities For the non tuberculous group the percentage of ACCF was 56 as against 51 for the tuberculous group For metaplasia and atypical metaplasia the

ratios were the opposite 70 and 46 per cent for the tuberculous patients as against 61 and 40 per cent respectively for the non tuberculous group None of these differences is however significant

When the figures are to be judged it is important to note that the percentage of heavy smokers i e ≥ 20 cigarettes a day was much higher among the smokers without lung diseases than among those suffering from inflammatory lung affections (Table 1)

DISCUSSION

As to the cytologic alterations studied in this investigation ACCF is interpreted as indicating injury to the respiratory epithelium The injury may be a starting point for the metaplastic process in the mucosa This damage and its consequences may be regarded as due to the action of exogenic factors which are not necessarily carcinogenic in a strict sense Naturally a true carcinogen may also cause injury to the epithelium and produce these alterations On the other hand the phenomena of cellular and nuclear atypia in the metaplastically changed mucosa indicate that a carcinogenic element is at least a part of the causative complex which produces the injury Atypical cell changes are nearly always found in metaplastic but not in normal respiratory epithelium This fact indicates that epithelium which is subjected to metaplastic alterations is more sensitive to carcinogenic action than normal epithelium Consequently factors which though non carcinogenic are injurious to the mucosa may facilitate the action of carcinogens

In this study the elements of exogenic infection which form the basis of the bronchial epithelial changes in the groups with inflammatory lung diseases are not carcinogenic as such With regard to tobacco smoke on the other hand there are convincing reasons for assuming that factors causing epithelial injury and metaplasia as well as carcinogenic elements are involved

The main purpose of this investigation was to study by sputum cytologic technique the importance of the combined action of a non carcinogenic causative complex *i e* chronic inflammation and a complex which includes a carcinogenic factor *i e* cigarette smoking Naturally it has not been possible to obtain groups for comparison which are completely free from objections There is among other things a carcinogenic component in the general air pollution which particularly affects the urban population In this connection it is of importance in the evaluation of the incidence that the groups of subjects without lung disorders were more urbanized than those in the inflammatory groups (Table 1)

The non smokers without lung diseases had a low rate of ACCF metaplasia and atypical metaplasia a result which could be expected The existence of single cases with epithelial atypia also in this group

may very likely be ascribed to the effect of the general air pollution all the subjects in this series were living in towns

It is of considerable interest that the smoking habit in the group without lung diseases led to a strong increase in the phenomena of ACCF which indicates epithelial injury and its consequence i e metaplasia Frequency here was only slightly lower than that in the group of smokers with chronic inflammatory disorders this group in turn exhibited an insignificantly lower frequency of ACCF and metaplasia than that in the group with established lung cancer The results may also indicate that heavy cigarette smoking in subjects without lung disease produces a substantially higher incidence of such epithelial lesions than does chronic inflammation alone i e in non smokers

The study demonstrates that the frequency of atypical (dysplastic) epithelial changes caused by heavy cigarette smoking in persons without lung affections is about the same as that caused by chronic inflammatory action alone on the respiratory mucosa A combination of the two factors smoking and chronic inflammatory mucosal action led to a markedly higher incidence of epithelial atypia Under these circumstances atypia reached the same level as that in the patients with established lung cancer The importance of the inflammatory co factor is emphasized by the fact that only a small number of the smokers in the inflammatory group were heavy smokers (Table 1)

Thus the results of this investigation have underlined that not only smoking alone which is already known but also chronic infectious lung disease in non smokers causes a distinctly injurious effect (ACCF and metaplasia) on as well as atypia of the respiratory mucosa

The results indicate that the effect of heavy smoking alone under the present conditions may be stronger than that of chronic inflammation alone Furthermore the study has proved that the combination of these two factors substantially augments the incidence of atypical metaplasia which in fact may be regarded as the true premalignant alteration

SUMMARY

The effect of cigarette smoking on the epithelium of the respiratory passages has been studied with sputum cytologic technique For this purpose we investigated a material consisting of non smokers and smokers without lung disease a material containing patients with chronic inflammatory lung disorders divided into smokers and non smokers and a bronchogenic carcinoma series in which all the patients were smokers

The frequency of abnormal columnar cell findings (ACCF) (17) metaplasia and atypical metaplasia (dysplasia) were recorded The series of established bronchogenic carcinoma presented the highest values for all three alterations and the group of non smokers without lung diseases exhibited the lowest

The distinct increase in the frequency of ACCP metaplasia and atypical metaplasia respectively in the group of smokers without lung diseases and in the group of non smokers with chronic inflammatory lung affections indicates that both cigarette smoking and chronic inflammation as such have a distinct injurious effect (ACCP and metaplasia) on the respiratory mucosa Damage to the epithelium due to heavy cigarette smoking alone seems to be more pronounced than that caused by chronic inflammation alone The frequency of atypical metaplastic changes produced by cigarette smoking alone and by chronic inflammation alone shows a high and equal level When these two factors are combined the frequency is greatly increased especially as regards the atypia phenomenon its incidence then reaches approximately the same high level as that in established bronchogenic carcinoma

REFERENCES

1 *Auerbach O Stout A P Hammond E C & Garfinkel L* Changes in Bronchial Epithelium in Relation to Cigarette Smoking and in Relation to Lung Cancer New Engl J Med *265* 253 267 1961

2 *Berkheiser S W* Atypical Bronchiolar Proliferation and Metaplasia Associated with Tuberculosis Dis Chest *45* 522–527 1964

3 *Campbell A H* The Association of Lung Cancer and Tuberculosis Austral Ann Med *10* 129–136 1961

4 *Campbell A H & Lee F J* The Relationship between Lung Cancer and Chronic Bronchitis Brit J Dis Chest 57 113–119 1963

5 *Case R A M & Lea A J* Mustard Gas Poisoning Chronic Bronchitis and Lung Cancer An Investigation into the Possibility that Poisoning by Mustard Gas in the 1914–18 War Might be a Factor in the Production of Neoplasia Brit J prev soc Med 9 62–72 1955

6 *Chang Suk Chul* Microscopic Properties of Whole Mounts and Sections of *Human Bronchial Epithelium of Smokers and Nonsmokers Cancer 10* 1246–1262 1957

7 *Cross K R Wol D V Palmer G K & Warner F D* A Study of the Tracheobronchial Epithelium and Changes Related to Smoking J Iowa State Med Soc p 137–140 March 1961

8 *Doll R & Hill A B* A Study of the Aetiology of Carcinoma of the Lung Brit Med J 2 1271–1286 1952

9 *Finke W* Chronic Pulmonary Disease as a Possible Etiologic Factor in Lung Cancer Int Rec Med 196 61–72 1956

10 *Garret Maria* Cellular Atypias in Sputum and Bronchial Secretions Associated with Tuberculosis and *Bronchiectasis Am J Clin Path 34 237–246 1960*

11 *The Health Consequences of* Smoking U S Department of Health Education and Welfare Public Health Service Publication No 1696 pp 87–124 1967

12 *Ide G Saint elf Valentina & Cowdry E V* A Comparison of the Histopatholog of Tracheal and Bronchial Epithelium of Smokers and Non Smokers Cancer *12* 473–484 1959

13 *Kawecka Maria* Cytologische Bewertung des Auswurf bei Patienten mit *Bron*chiectasen und die Moglichkeit einer karzinomfehldiagnose Acta Unio Int. Contra Cancrum *15* 469–473 1959

14 *Knudtson K P* The Pathologic Effects of Smoking Tobacco on the Trachea and Bronchial Mucosa *Am J Clin Path 33 310 317 1960*

15 *Nasiell M* The General Appearance of the Bronchial Epithelium in Bronchial Carcinoma A Histopathological Study with Some Cytological Viewpoints Acta Cytol *7 97–106 1963*

16 *Nasiell M* Metaplasia and Atypical Metaplasia in the Bronchial Epithelium A Histopathologic and Cytopathologic Study Acta Cytol *10* 421–427 1966

17 *Nasiell M* Abnormal Columnar Cell Findings in Bronchial Epithelium A Cytologic and Histologic Study of Lung Cancer and Non Cancer Cases Acta Cytol *11* 397–402 1967

18 *Nasiell M* Comparative Histological and Sputumcytological Studies of the Bronchial Epithelium in Inflammatory and Neoplastic Lung Disease Acta path microbiol scandinav 72 501–518 1968

19 *Robbins W T* Bronchial Epithelium in Smoking and Non Smoking College Students J Am Coll Health Ass *14* 265–266 1966

20 *Sanderud K* Squamous Metaplasia of the Respiratory Tract Epithelium. 2 Relation to Tobacco Smoking Occupation and Residence Acta path micro biol scandinav 43 47–61 1958

21 *Smoking and Health* U S Department of Health Education and Welfare Public Health Service Publication No 1103 pp 263–313 1964

22 *van der Wal A M Huizinga E Orie N G M Sluiter H J & de Vries K* Cancer and Chronic Non specific Lung Disease S and J resp Dis 47 161–172 1966

Acta path. et microbiol. scandinav. 74: 214–222, 1968

The University Laboratory of Neuropathology (Head: Erna Christensen †) and the
University Institute of Pathological Anatomy (Head: C. Teilum)
Copenhagen, Denmark

EXTRAGONADAL ENDODERMAL SINUS TUMOURS ORIGINATING IN THE REGION OF THE PINEAL GLAND

By

JOHN BESTLE

Received 19.iv.68

The endodermal sinus tumour as defined by Teilum (14, 15) is a characteristic type of germ cell tumour showing a selective overgrowth of yolk sac endoderm associated with extra-embryonic mesoblast. These tumours in the ovary and the adult testis were assumed to arise from totipotent germ cells and their germinal origin was confirmed by the occasional presence of areas of germinomatous, teratoid or trophoblastic structures.

Histogenetically the interpretation of endodermal sinus tumours in *the ovary was based on*

1. their histological identity with "embryonal" tumours seen in rare cases in the adult testis (12),
2. the loose reticulated meshwork analogous with the extra-embryonic mesoblast (13) and the characteristic perivascular structures formed by invaginated blood vessels mantled by yolk sac endoderm (endodermal sinus) (14, 15).

Endodermal sinus tumours are in all cases seen to occur in young patients; in cases in which the tumour originated in the ovary, the age of patients ranged between two years and a half and 35 years (8), most cases occurring in patients in the age group 16–25 year. The histogenesis of a characteristic type of testicular tumour in early childhood has been an object of much discussion and various opinions have been advocated; it has been interpreted as a distinctive adenocarcinoma of infantile testes, attributable to non-germinal parenchyma (16), but later it has been classified as a germinal tumour exhibiting the endodermal sinus pattern described by *Teilum* (2).

Of great interest are recent reports of cases of typical endodermal sinus tumours originating in extragonadal areas where other types of germinal tumours are known to develop. *Rao Raghunatha et al.* (1964) found the tumour to be associated with the sacrococcygeal teratoma

and *Teilman et al* (1967) have reported the first case of an endodermal sinus tumour arising in the anterior mediastinum in a 31 year old male patient

In this connection the present report of two cases of endodermal sinus tumours of intracranial origin in the region of the pineal gland may contribute to the understanding and proper identification of germ cell tumours orginating in extragenital sites

CASE REPORTS

Case 1 The patient was a 13 year old girl who had been premature Since the age of three years she had worn glas e on account of hypermetropia and strabismus Throughout the last five years episodes of a ri ht sided migrainoid headache had occurred for the last three years accompanied by progressing loss of sight For these reasons the patient was hospitalized about two years ago on that occasion arteriography and pneumography had excluded the presence of a tumour By cranio tomy combined with chiasma/exploration the arachnoides was found to be of a viscous consistency but there was no tumour growth The optic nerve to the left was very thin its diameter measuring only half that of normal nerves Gross inspection of the optic nerve to the right showed normal conditions The patient wa discharged but she continued to visit the eye department for follow up quite recently she has been re admitted be ause her sight gradually had deteriorated ensibility to light of the right eye lacked During the last months preceding hospitalization the visual power of her left eye had also abated As before episode of intermittent migrainoid headaches above the right eye continued

At a sub equent examination the girl was found to suffer fr m moderate dys crinism and the endocrinological examination sugge ted a le ion of the hypothalamus Hgb 12 5 g/per cent SR 10 mm metabolism 103 per cent Examination of the eyes disclosed that the pupillae were large but of rather unif rm size The reaction to light of the left eye was normal but the right eye lacked ensibility to light *Visual* power of the left eye ranged at 1/60 On either side the papillae of the optic nerve were severely atrophic By roentgen examination of the skull the dor um sellae were found to be destr yed Findings by air encephalography included anomaly *of the interp dun ular* cisterna and horizontal severance at the level f the dorsum sellae By right sided carotis arteriography a large arcuate elevation of the *horizontal p rtion of both* of the anterior cerebral arteries was noted At the subsequent operation a large encapsulated and well defined tumour was ob erved behind the *anterior clinoid process* The right optic nerve was completely destro ed while the left one which pa sed through the tumour was swollen oedematous and embedded in arachnoidal adheran e At the crossing of the chiasma the optic nerve was seen to m rge with the tumour and consequently it could not be dissected Cutting through *the removed* tumour revealed a soft yello i h tissue of glious aspect without cy t formation

The patient was discharged and transferred to the Radium Centre fo roentgen treatment

Case 2 The second *patient* was an 11 year old boy who throughout the last 18 months preceding ho pitalization had complained of o ca ional headaches localized to the forehead *for the last month* the headaches has intensified and a psychi hang had set in the bo had become very quiet and testy and he wanted to have a daily rest *During the last week before* hospitalization the patient had complained of diplopia and also of a certain d zzin ss There was nausea but vomiting did not occur At the sub equent examination the visual power of both eyes was f und t be normal there was a ce tain instability of the eye mu cles together with arying degrees of abductor *paralys s most* pronounced on the left side intense adduction ny tagmus and paralysis of the conjugate (upwards) movement of the eye The isual field was normal *Ophthalmoscopy* showe l normal excavation of the optic nerve margins towards the nose were rather blurred and on the left si le dubious venous pulse was f und

In the evening of the day of hospitalization the boy became suddenly ill and gradually the state of consciousness abated Pareses did not set in but a bilateral Babinsky reaction was manifest In front of and above the pineal body a calcified process was found by radiography Arteriography revealed the elevated position of the vena galeni Posteriorly the last half cm of the latter was not filled which feature also applied to the sinus rectus The central veins were displaced a little to the right of the midline Performance of preliminary drainage of the ventricles improved conditions to a certain degree Pneumo encephalography disclosed a peculiar humpy configuration at the site of the pineal body At operation performed by two steps a large solid tumour was found to invade the posterior portion of the left ventricle continuing diffusely downwards into the brainstem A biopsy from the tumour showed the presence of a malignant mixed tumour Radical operation was impossible Post operatively a rapid aggravation of the condition set in death occurred on the following day

At autopsy a large ovoid tumour measuring 6 × 4 × 4 cm was found to fill completely the third ventricle growth continued into the lamina quadrigemina compressing the aqueduct Splenium corporis callosi was destroyed ventrally the body was compressed and partially invaded by the tumour The thalamus in either hemisphere and the caudal portion of the corpora striata were invaded The tumour tissue was of a greyish colour it was of a solid consistency and moderately mottled by small haemorrhagic strands Caudally to the tumour at about the site of the pineal body a spherical well defined area measuring less than 1 cm was found to contain sebaceous material and hair Sagittal section through both side ventricles showed that the tumour growth extended right to this site The extracranial autopsy displayed nothing pathological except hyperaemia of the lungs The gonades were normal

Pathology

At the gross inspection the two tumours were found to be different in the first case the tumour was partly well defined and encapsulated whereas the tumour in the second case was characterized by infiltrative and erosive growth Furthermore the localization of the two tumours was different although they in either case had developed in regions generally known to be sites of teratomas and pinealomas

Histologically however the two tumours were almost identical presenting the characteristics on which *Teilum* based the diagnosis of endodermal sinus tumour these characteristics (15) include

1 Areas of stellate cells arranged in a loose vacuolated network composed of wide meshes or cystic cavities often lined with flat (mesothelial) cells and containing foci with active haemopoiesis in the underlying capillary cavities (Fig 7) Many meshes contain hyaline acidophile and PAS positive globules (Fig 8) or mucoid deposits In more compact areas star like halos of cells surround the larger capillaries
2 Peculiar perivascular formations formed by invaginated mantled blood vessels and recapitulation of the embryologically well defined endodermal sinuses are characteristic The surrounding capsular space is lined by a single layer of flat cells with prominent dark nuclei (Figs 1 2 3 and 4)
3 The small aggregates of undifferentiated neoplastic embryonal cells are less characteristic they should rather be considered



Figs 1–2

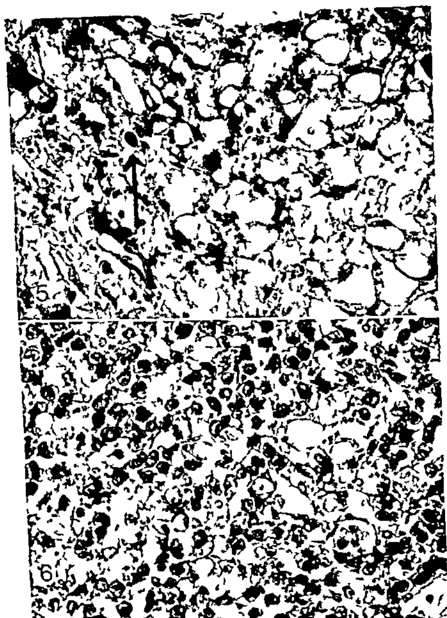
Fig 1 The endodermal sinus (case no 1) H-E 560 ×
Fig 2 The endodermal sinus (case no 1) H-E 560 ×



Figs 3–4

Fig 3 An area with numerous endodermal sinuses in cross section (case no [illegible]) H–E. 140 ×

Fig 4 Endodermal sinuses and small and larger vesicles (case no [illegible]) H E. 350 ×



Figs 5–6

Fig 5 Intra and extracellulary PAS positive globule (arrow) PAS 350 ×
Fig 6 A solid dysgerminomatous part of the tumour (case no 1) H–E 350 ×

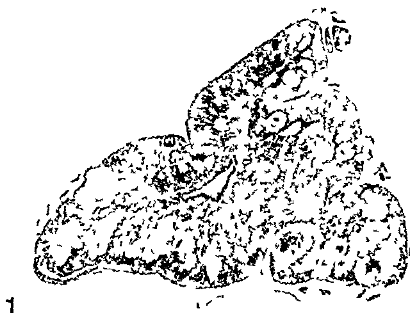


Fig 1

Adrenal with necroses Van Gieson Hansen Stain mag 6 ×

by the following reactions Anti streptococcus MG anti mycoplasma pneumoniae *influenza B and D titres* Toxoplasmosis anti streptococcus hyaluronidase Bunnell s and Widal s *reactions* were negative ESR [illegible]–38 mm/h Haemoglobin 11.5–14.0 g/100 ml leucocytes 9000/µl normal differential *count thrombocytes* 233000 µl *Prothrombinindex 90 per cent bleeding and coagulation time 5 minutes In the* serum complete and incomplete leucocyte agglutinins were found but *no erythro*cyte or thrombocyte antibodies were present Listeria serology was not performed Listeria could not be cultured from vagina four months after the delivery

The infant was born asphyxiated He did not *cry there was only superficial* respiration after some minutes There was severe cyanosis and hypotonicity and scattered petecchiae grew to ecchymoses Heart rate was 60/min

He was continuously ventilated manually initially on a mask later on through a tracheal tube A bloodtransfusion was given into the umbilical vein but the infant expired four hours after birth

Laboratory data Haemoglobin 10.9 g/100 ml reticulocytes 44 per thousand thrombocytes 80000/µl leucocytes 13700/µl differential count No granulocytes lymphocytes 84 per cent leucocyte precursers 8 per cent monocytes 8 per cent Serumbilirubin 2.5 mg/100 ml Blood group A D positive Complete leucocyte agglutinins were not demonstrated

Necropsy A premature pale boy with many cutaneous petecchiae on the extremities *A moderate mediastinal emphysema* was found On the surface and in the parenchyma of the liver wide spread tiny whitish yellow foci were seen In the adrenals *these foci were abundant (Fig 1)* The other organs showed no alterations

Brain autopsy A slight blurring of the leptomeninges was seen on the basis Coronal sections *through the cerebrum revealed a minor hae*



Fig 2 [illegible] with [illegible] reaction Haematoxylin eosin stain ×

Fig 3 [illegible] ed [illegible] adrenal No granulocytes Gram ×

morrhage around a terminal vein The semiovale centre had lowered consistency and was slightly discoloured The brain stem spinal cord and cerebellum were of normal appearance

Bacteriology Tissue specimens from the organs were sent to bacteriogical examination Numerous Gram positive bacteria were erroneously diagnosed as enterococci and Group B haemolytic streptococci at the routine examination No special culture *for Listeria was performed*

Histology In the lungs a few small nodular necroses with only a small amount of mononuclear cells and no granulocytes were found *In the necroses only one kind of bacteria* was seen Short Levaditi positive and Gram positive rods In the liver (Fig 2) and the adrenals nodular necroses were abundant Here a large number of bacteria was found The bacteria were of the same appearence as in the lungs occuring singly in small crowds or short chains or in angle formations (Fig 3) In the spleen some and in the kidneys very few necroses were seen In the myocardium the thyroid gland thymus pancreas and testes were no processes

Within the red bone marrow from decalcified lumbar vertebrae scattered necrotic foci with the same bacteria were found There was no fibrosis and there were no precursers of granulocytes

A sternal smear taken shortly before the infant died showed abnormal granulopoiesis Myeloblasts promyelocytes and myelocytes showed large forms with coarse vacuoles in nuclei and cytoplasm These *cells were found in approximately normal number and no rod shaped* or segmented polymorphonuclear granulocytes were seen The eosinophilic cells showed normal forms The erythropoiesis was rather pronounced and normoblastic

Peripheral blood showed no granulocytes but many lymphocytes and in addition some nucleated red cells

Brain histology In the leptomeninges of the brain and spinal cord there were focal infiltrations with inflammatory cells among which some were polymorphonuclear predominantly eosinophilic granulocytes and some were large mononuclear cells At this sites were also crowds of bacteria Most nerve cells in the cerebral cortex appeared either as severely pycnotic cells or as shadow cells In the cerebellar cortex the lack of Purkinji cells was outstanding In a few places of the ovale centre streaks of rod shaped bacteria were found and in the *surroundings* there was glial proliferation but no inflammatory cells Only in the basal ganglia and the brain stem a few foci with hyperaemia bacteria and infiltrating mononuclear cells were seen but no *granulocytes*

Placenta Some villi were infiltrated with polymorphonuclears the throphoblasts were degenerated and there was fibrin on their surface Bacteria of the same *morphology as in the organs* and the brain were seen here No bacteria or inflammatory cells were found in the membranes or in the umbilical cord

Diagnosis Listeria sepsis Granulomatosis infantiseptica? Meningo encephalitis Neutrophilic granulocytopenia Placentitis

Epicrisis During the 32nd week of pregnancy a 25 year old previously healthy woman had influenza like symptoms for which she received 3 5 g of sulphadimethoxium She recovered in 7 days One month before term she gave birth to a premature male infant There was a rise in temperature during delivery which was otherwise uncomplicated The infant was extremely pale with respiratory insufficiency and petecchiae of the skin appeared Examination of peripheral blood and bone marrow showed agranulocytosis The infant died 4 hours after the delivery At autopsy numerous minor necroses were found in the liver and adrenals Microscopy showed myriades of bacteria of one type Short Gram positive argentaphilic rods arranged in small groups short chains or single formations The same bacteria were seen in microscopic necroses in the lungs and spleen and focally in the leptomeninges and the cerebrum Also in the bone marrow which showed a decreased maturation of neutrophile granulocytes the bacteria were found In the placenta inflamed villi with the same bacteria and necroses were seen

DISCUSSION

The diagnosis of listeriosis was not verified bacteriologically But according to *Seeliger* (1961) it is possible to make a strongly suggestive diagnosis on morphological grounds *i e* by the characteristic pathological anatomical picture of granulomatosis infantiseptica with organ necroses especially in the liver spleen and adrenals together with meningitis and Gram positive argentaphilic short rod shaped bacteria within the necroses

The route of infection was probably transplacental The rupture of the membranes occured immidiately before the delivery In the placenta inflamed villi with bacteria of the same type as those present in the organs of the infant were seen These observations make it probable that the infant was infected haematogeneously through the placenta The minor degree of infiltration in the lungs in comparison with other organs favours this assumption The massive organ necroses are in accordance with the assumption that the infant was infected some time before the delivery

Examination of the sternal marrow aspirate and peripheral blood taken shortly before the infant s death showed neutrophilic agranulocytoses Postmortem microscopy revealed a moderate number of polymorphonuclear granulocytes in the leptomeninges In the organ necroses granulocytes were absent

The question now is whether our case represents an infantile genetically determined agranulocytosis (primary bone marrow insufficiency) is due to an immunologic transplacental reaction to a drug induced transplacental reaction or to a bacterio toxic influence

Kostmann (1956) described the infantile genetically determined agranulocytosis He found six cases in a population in which the inci dence of intermarriage was high and concluded that it was an auto somal recessive condition During the first weeks or months of life the infants developped skinsepsis *Aarskog* (1961) found myelofibrosis and absence of neutrophilic *myelogenic elements of the bone marrow* in a few cases *MacGillivray et al* (1964) described five cases all compli cated with a not congenital sepsis In our case there was no consangui nity or family history of similar cases There was no myelofibrosis and the sepsis was of intrauterine origin

In immunological leucopenia leucocyte agglutinins of identical types are found in the mother s and the infant s serum This isoimmunization of the mother is seen in rare cases after pregnancies and transfusions (Stephanini 1958 Gert Jensen 1960 and Halvorsen 1965) One of the newborn infants described died from malformations the others were leucopenic but recovered Agglutinins were found in the serum of most *but not of all the infants In our case* the serum of *the mother* contained both complete and incomplete *leucocyte* agglutinins She had no leuco penia The serum of the infant was only examined for complete agglu tinin and this was not found

Several drugs including sulphonamides may provoke leucopenia in adults Sulphonamides pass quikly through the placenta (*Sperrt 1940*) and their hepatotoxic and enzymeblocking properties in the human foetus are well known (*Gin ler* 1942) To our knowledge there is only one report of a destructive effect of the drug on the bone marrow (*Stamm et al* 1965) In that case the mother had been treated for an urinary infection with 210 Gantrisin(R) tablets (Sulphixalol) from the 23rd to the 32nd week of pregnancy In our case *the mother had had* 7 Madribon(R) tablets in the 32nd week

The histological findings of foci with bacteria in the red bone marrow in our case might indicate a direct bacteriotoxic influence on the haemo poietic tissue To our knowledge however congenital agranulocytosis secondary to bacterial infection has not been reported Leucopenia in the post natal period caused by coli and streptococcal sepsis has been described (*Nyhan* 1958 *Clifford* 1947) Among patients with listeriosis after the neo natal period a few cases have been found to have a slightly reduced number of granulocytes in most cases the granulocyte count was normal and in some cases there was granulocytosis (*Toda & Wood Sell* 1963 *Ray & Wedgwood* 1964 and *Insley & Hussain 1964*) Among eleven cases of listeriosis *Hauge Kristensen & Jessen* (1960) found one patient with pronounced granulocytopenia and two with some periphe ral granulocytopenia Three of the patients were newborn infants but none of these were described as neutopenic

Among the four above mentioned possible aetiological mechanisms it is not possible to decide which one was encountered in this case

SUMMARY

A case of morphologically proven but not bacteriologically verified congenital Listeria sepsis in a premature male infant who died four hours after delivery is reported The mother had had an uncharacteristic illness with fever treated with sulphonamide one month before the delivery The infant had a pronounced neutrophilic granulocytopenia in the peripheral blood and bone marrow as well as organ necroses The different forms of granulocytopenia in newborn infants are mentioned and the possible relation between listeriosis and granulocytopenia is discussed

ADDENDUM

Since the manuscript was submitted the mother gave birth to her third infant twenty months after the infant described in the case report The latest pregnancy was uneventful with normal blood counts Several cultures of the vaginal secretion failed to show Listeria The delivery was uncomplicated and the infant (a female birthweight 3100 g) thrived well

REFERENCES

Aarskog D Infantile congenital agranulocytosis Arch Dis Childh 36 511–514 1961

Clifford S H Discussion on infection in the newborn peri d. J Pediat 30 696–698 1947

Gin ler A M & Chesner C Toxic manifestations in the newborn infant following placental transmission of sulfonamide Amer J Obstet & Gyn 44 46–49 194

Halvorsen K Neonatal leucopenia due to fetomaternal leucocyte incompatibility Acta Paed Scand. 54 86–90 1965

Insl y J & Hussain Z Listerial meningitis in infancy Arch Dis in Childh 39 278–28(1964

Jens n K Cerl Transplacental passage of leucocyteagglutinin occurring on account of pregnancy Dan Med Bull 7 55–58 1960

Kostman R Infantile genetic agranulocytosis Acta Paediat (Stockholm) Suppl 105 1956

Krist nsen K Hauge & Jessen O Forel omst af human listeriose i Danmark Ugeskr f L 122 127–135 1960

Loda F & Wool Sell S H Meningitis in newborn infants due to Listeria monocytogenes Southern Med. Journ 6 872–875 1963

Ma Gillivray J B Dacie J V Henry J R.K Sacker L S & Tr ard J P M Congenital neutropenia. Acta Paed 53 188 203 19 4

Murray E C D Webb R A & Swann M B R A disease of rabbits characterized by large mononuclear leucocytosis caused by a hitherto undescribed bacillus Bact monocytogenes J Pathol Bacteriol 29 407–439 1926

Nyf III A Etiologie de la mononucleose infectieuse Compt Rend Soc Biol *101* 590 591 1929

Nyhan W L & Fous l M D Septicem a of newborn Pediatrics 2 268–278 1958

Ray C G & Wedgwoo l R J Neonatal listeriosis six case reports and a review of the literature Pediatrics 34 378–392 1964

Se l g r H P R Listeriosis S Karger Basel New York P 308 19 1

Seelig r H P R Die atiologische Diagnose der Listeriose *Zentrallblatt fur Bakt* Parasitk Infektionskrank & Hygiene *187* 267–27(19 2

Sper rt H The passage of sulfonilamide in the human placenta Bull Johns Hopk H p 66 139–155 1940

Stamm O Sr benmann R Bigler R & Flury R Connatale Agranulocytose Gynaec logia 159 265–276 1965

St fania M M le R H & Skinner D Transitory congenital neutropenia A new syndrome Am. Journ Med 2 749 758 1958

W nblal S Studies of antibodies in human listerios s 1 Acta path microbiol scandinav 59 1 3–132 1963

Acta path et microbiol scandinav 74 230-236 1968

The Bacteriological Department of Serafimerlasarettet Stockholm Sweden
(Head Arne Lithander M D)

DEVELOPMENT OF TUBERCULOUS ALLERGY AFTER INOCULATION WITH BCG VACCINE IN OFFSPRING OF FEMALE GUINEA PIGS INOCULATED WITH BCG VACCINE

By

ARNE LITHANDER

Received 9 iii 68

The effect of passive immunity from the mothers on the vaccination response in infants and newborns has attracted increasing attention in recent years

Many investigators have found that in infants antibodies transferred from the mother during pregnancy interfere with the vaccination with several bacterial antigens *Vahlquist* (1) observed that the formation of circulating antibodies to diphtheria was depressed in infants who had circulating diphtheria antitoxin in serum passively received from the mother with a level of approximately 0 02 U/ml *Osborn et al* (2) confirmed these results but their data seemed to indicate a slightly higher critical level than that found by *Vahlquist*

Brown et al (3) and *Spigland et al* (4) stated that in the newborn period the presence of maternally aquired antibodies was associated with a low serologic response in the infant s serum to immunization with polio vaccine *Gaisford et al* (5) considered immunization in the newborn period with polio vaccine to be impracticable chiefly because of inhibition by placentally transmitted antibodies Similar results have also been found with other bacterial vaccines

In experimental investigations *Kosunen* (6) found that the response of young mice to tetanus immunization was clearly depressed by antibodies from the mother

In BCG vaccination the presence of tuberculin allergy is generally accepted as a criterion of resistance The Mantoux conversion is generally accepted as a proof of tuberculous allergy

After BCG vaccination it is often found that the tuberculin reaction remains negative or that Mantoux conversion takes place only after a very long time This applies particularly to vaccinated newborns and infants From many investigations it seems that after BCG vaccination of the mother also a factor transferred from her inhibits the conversion to tuberculin sensitivity in the offspring

Turell (7) and *Rollof & Lagercrantz* (8) found that after BCG vaccination the tuberculin reactions were positive in the children of healthy mothers earlier than in those of tuberculous mothers. *Hagberg* (9) vaccinated newborns of tuberculous mothers. He found that BCG tuberculin (Calm) gave a positive reaction earlier than Old Tuberculin (OT). Using both tuberculins the percentage of positive reactions was found to be higher in the children of healthy mothers than in the children of tuberculous mothers. *Hagberg* ascribed this difference to a factor possibly with the nature of an antibody transferred in utero from the tuberculous mother.

Weiss (10) and *Weiss et al* (11) injected OT and BCG bacilli both into guinea pigs in utero and into newborn guinea pigs. Subsequently injections of these antigens were given to the same animals some time after birth. Subsequent tuberculin tests on these animals were largely negative. *Weiss* considered that this was due to the fact that the first injections of antigen made the animals tolerant to the antigens.

In investigations on guinea pigs *Lithander* (12) found in tests both with Calm and with OT that the frequency of positive reactions in the young of healthy females was higher than in the young of tuberculous females after BCG vaccination. The results seemed to be in agreement with observations made in investigations on children of tuberculous and of healthy mothers. The results were interpreted as being due to an interference by a factor transferred from the tuberculous mother. The interference was mainly apparent in tests with OT and decreased with age at vaccination.

To complete my work on this subject I investigated by means of intracutaneous tuberculin tests whether infection with BCG bacilli in female guinea pigs before impregnation would have an effect on the Mantoux reaction of their offspring.

MATERIALS AND METHODS

Preparation and Testing of BCG

Calm was prepared from BCG cultures on Sauton's medium for the production of BCG vaccine in the same way as described earlier (12). This description applies to the titration of the potency *of the Calm* compared with a particular batch of OT and to the innocuity tests.

Animal Experiments

The tests were performed on the offspring of white female guinea pigs. The female guinea pigs had been infected with an injection of 15 mg of BCG bacilli. Five weeks later it was established by means of intracutaneous tuberculin tests with 0.5 mg of OT and 0.5 mg *of Calm that the* infection had produced allergy in the animals. The guinea pigs were impregnated immediately after positive reactions to these tests had been obtained. Their offspring were vaccinated with 5 mg of BCG vaccine at the ages of 2, 4, 6, 8 and 10 weeks in different series respectively according to age when vaccinated. The number of animals in each series is shown in Table 1.

The above mentioned investigation was performed at the same time as the investigation using BCG vaccination in the offspring of tuberculous female guinea

pigs (12) The same batches of Calm and OT respectively were used in both investigations

TABLE 1
Number of Offspring of BCG infected Female Guinea Pigs Vaccinated with 5 mg of BCG Vaccine at Different Ages

Age at BCG vaccination in weeks	Number of offspring
2	48
4	48
6	41
8	64
10	67

Table 1 includes only those animals in which all tuberculin tests were invariably performed two to eleven weeks after vaccination

The presence of tuberculin allergy was ascertained by intracutaneous injections of 1 0 and 0 5 mg of OT in the shaved dorsal skin and of equivalent doses of Calm 2 3 4 5 6 and 11 weeks after vaccination in all the animals The tests were read after 48 hours An area of induration of a least 10 mm in diameter was considered a positive reaction

RESULTS

Fig 1 illustrates the cumulative percentages of positive reactions to tests with doses of 1 0 and 0 5 mg of Calm and OT respectively from two to eleven weeks after BCG vaccination in the offspring of BCG infected female guinea pigs

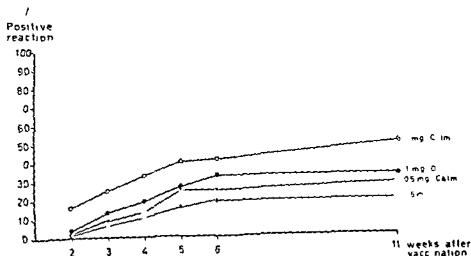


Fig 1

Cumulative percentages of ... ive reactions to 1 0 and 0 5 mg of Calm and to 1 0 and 0 5 mg of OT respecti... ifferent intervals after vaccination with 5 mg of BCG vaccine of the off... CG infected female guinea pigs The young were v... the age ... 10 weeks

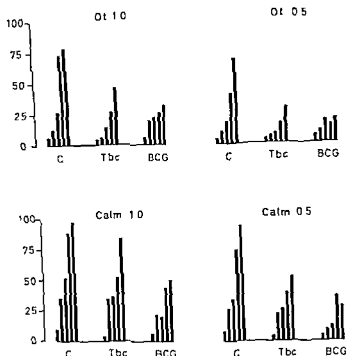


Fig 2

The frequency of positive tuberculin reactions tested with 1 0 and 0 5 mg of OT and Calm respectively which developed during 11 weeks following vaccination of young guinea pigs at varying ages (cumulative percentages) The figures of the vertical axis denote percentages of positive tuberculin reactions The figure under each group of piles denotes the age in weeks at vaccination The first pile in each of these groups denotes the young of healthy the second of tuberculous and the third of BCG infected female guinea pigs

The figure shows that the frequency of positive reactions was low during the first weeks after vaccination but increased successively with the interval after vaccination This applied to Calm as well as to OT The same tendency corresponded roughly with that in the off spring of healthy and of tuberculous females (12)

A marked effect of the age at vaccination was found by comparing the results in animals at different ages at vaccination There were no positive reactions in the young vaccinated at two weeks until eleven weeks after vaccination The increase of positive reactions began earlier irrespective of the type of tuberculin in the groups in which vaccination ages were higher This increase rose with the age at vaccination At all intervals after vaccination the frequency of positive reactions obtained with Calm was slightly higher than that obtained with OT This tendency was largely the same with both doses of tuberculin

In Figs 2 and 3 the results of this study are also compared with

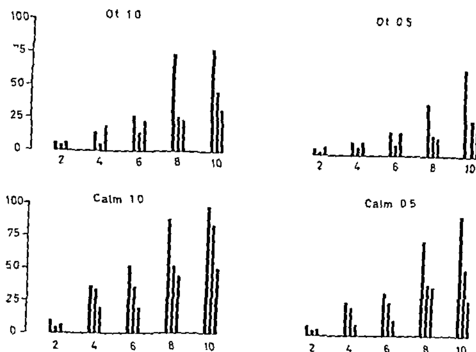


Fig 3

The frequency of positive tuberculin reactions tested with 1 0 and 0 5 mg OT and Calm respectively which developed during 11 weeks following vaccination of young guinea pigs at varying ages (cumulative percentages) The figures of the vertical axis denote percentages of positive tuberculin reactions Under the groups of piles C denotes the young of healthy TBC of tuberculous and BCG of BCG infected female guinea pigs In each group the piles denote from left to right the vaccination ages of 2 4 6 8 and 10 weeks

those obtained in an earlier investigation (12) of the offspring of healthy females and of females infected with virulent bovine tubercle bacilli (tuberculous females)

These figures show that the frequency of positive reactions to doses of Calm of both 1 0 mg and 0 5 mg was higher in the offspring of healthy females than in those of BCG infected females from the vaccination age of four weeks The difference increased with the vaccination age When the vaccination age was 8 and 10 weeks the frequency of positive reactions obtained with OT was higher in the offspring of healthy females than that obtained in the offspring of BCG infected ones At these two vaccination ages 1 mg of OT gave an even higher frequency of positive reactions in the young of healthy guinea pigs than the same dosage of Calm produced in the young of BCG infected females

When the results obtained by OT in the young of BCG infected and of tuberculous females were compared the frequency of positive reactions in the offspring of BCG infected females tended to be higher than that seen in offspring of tuberculous females when the vaccina

tion ages were from 2 to 6 weeks At the vaccination ages of 8 and 10 weeks the differences seemed to be inverted Using Calm there was a tendency to a higher frequency of positive reactions in the offspring of tuberculous female guinea pigs than in offspring of BCG infected females when the age of infection was four weeks or more This applied to doses of both 1 0 and 0 5 mg of tuberculin

DISCUSSION

The present investigation clearly indicates that the ability to react with tuberculin produced by BCG bacteria was weaker in BCG vaccinated offspring of female guinea pigs which had been infected with BCG bacteria before impregnation than in offspring of healthy guinea pigs

This observation corresponds with what has previously been found in the offspring after BCG vaccination of tuberculous female guinea pigs (12) and also in newborns and infants of tuberculous mothers (9) and after vaccination with other bacterial antigens as well In this investigation it is quite reasonable also to consider the results due to a factor possibly with the nature of an antibody transferred from the infected female guinea pigs to their offspring This factor is supposed to have an inhibiting effect on the Mantoux reaction

The circumstances that the frequency of positive reactions increased and that a change to positive reactions came earlier at increased age at vaccination may indicate that the influence from the mother decreased with time Further evidence that this might be the case is that the frequency of positive reactions obtained with Calm was higher than reactions to obtained with OT in the offspring of BCG infected females when the age at vaccination was 8 and 10 weeks (Fig 2 and 3)

For a better understanding of the significance of the results in the investigations in question, a comparison was made between the results of corresponding studies on offspring of healthy female guinea pigs and of those infected with virulent tubercle bacilli (12)

Whether OT or Calm were used the frequency of positive reactions was higher in the offspring of healthy female guinea pigs than in offspring of females infected with BCG This difference was anticipated and is considered to be due to interference from an antibody derived from BCG infected mothers The circumstance that the difference was approximately the same whether OT or Calm were used in the tests might indicate that Calm and OT are similar antigens A comparison between the results in offspring of females infected with virulent tubercle bacilli and those of females inoculated with BCG bacilli reveals however that a higher frequency of positive reactions was obtained with Calm in the former than in the latter The contact with the antigen BCG bacilli *in utero* thus appeared to lead to an interference of the Mantoux reaction to Calm after the immunization with BCG vac

cine that was of a higher degree than the one seen after infection with virulent tubercle bacilli

SUMMARY

Mantoux tests were made on offspring of BCG infected female guinea pigs

The infection of the female with BCG bacilli led to a delay of the Mantoux conversion with BCG tuberculin (Calm) in the offspring when they were vaccinated with BCG vaccine The influence from the mother also reduced the frequency of positive reactions with this tuberculin

These effects were ascribed to a factor possibly with the nature of an antibody transferred in utero from BCG infected mother This interference varied with the age at vaccination and could be demonstrated also if old tuberculin were used

REFERENCES

1 *Vahlquist B* Studies on Diphtheria Decrease of Natural Antitoxic Immunity against Diphtheria Acta Pediat 35 117-129 1948

2 *Osborn J Dancis J & Julia* Studies of the Immunology of the Newborn Infant Pediatrics *10* 328-334 1952

3 *Brown G C Kendrick P I* Serologic Response of Infants to a Multiple Vaccine for Simultaneous Immunization Against Diphtheria Pertussis Tetanus and Poliomyelitis in Relation to the Presence of Specific Maternal Antibody Am J of Publ Health *50* 1529 1538 1960

4 *Spigland I Goldblum N* Immunization of Infants with Formalinized Poliomyelitis Vaccine (Salk type) Pediatrics 25 812-821 1960

5 *Caisford W Feldman G V Perkins F T* Current Immunization Problems J of Pediatrics *56* 319-330 1960

6 *Kosunen T Halonen I* Response of Young Mice of Immunized Parents to Tetanus Toxoid Ann Med Exper Fenn 38 [illegible] 1960

7 *Turell I* Antibodies to Tubercle Bacilli and Tuberculin Response after BCG vaccination in Infants of Mothers with Tuberculosis *Nord Med 52 1139* 1140 1954

8 *Rollof S J Lagercrant R* Antikroppar till tbb och tuberkulinreaktioner efter BCG vaccination hos barn till tuberkulosa modrar *Acta Ped 43 Suppl* 100 179-182 1954

9 *Hagberg E* Tuberkulinreaktionen hos spadbarn til tuberkulosa modrar Nord Med 52 1956-1957 1954

10 *Weiss D W* Inhibition of Tuberculin Skin Hypersensitivity *in Guinea Pigs by* Injection of Tuberculin and Intact Tubercle Bacilli during Fetal Life *J of Exp Med 108* 83 104 1958

11 *Weiss D W Wells A Q* Actively Acquired Tolerance to Tuberculo Protein Nature *179* 968-969 1957

12 *Lithander A* Development of Tuberculous Allergy after Inoculation with BCG Vaccine *in* Off pring of Healthy and Tuberculous Female Guinea *Pigs* Acta path microbiol scandinav *49* 165-1[illegible] 19[illegible]0

Acta path et microbiol scandinav 74 237-240 1968

Institute of Medical Microbiology University of Uppsala Uppsala Sweden

FURTHER STUDIES ON THE STAINING OF TUBERCLE BACILLI

By

VILHELM HALLBERG

Received 23 iii 68

Since the description of the Nachtblau method for staining acid fast microorganisms appeared (*Hallberg* 1946) this procedure has been used routinely as one of the diagnostic staining methods for tubercle bacilli During the course of its use problems have been met which have led to modifications of the original method

It should be stressed that there are different kinds of Nachtblau some of which give good results while others do not In our work we have preferred the following mark Nachtblau (Night Blue Bleu de Nuit) Chroma Gesellschaft Schmidt & Co Stuttgart Unterfurkheim A description of the staining procedure is given below

Staining Solutions Etc

Stock solution A 5 g of Nachtblau is dissolved in 100 ml of 96 per cent ethanol The flask is shaken repeatedly during the day left at least overnight for sedimentation of any possible undissolved material and filtered through a filtering paper

Stock solution B 4 g K_2SO_4 is dissolved in 100 ml of hot distilled water

The *Nachtblau staining solution* is prepared by mixing 90 ml of distilled water 1 ml of stock solution B and 10 ml of stock solution A

Decolorizing solution A 9 per cent nitric acid

Decolorizing solution B 70 per cent ethanol

Counterstain A 0 25 g of pyronine is completely dissolved in 100 ml of distilled water after which 0 5 g of phenol is added

Counterstain B 0 175 g of chrysoidine is dissolved in 200 ml of distilled water under boiling After cooling it is passed through a filtering paper

Simultaneous Decolorizing and Counterstaining Nitric acid followed by 0 5 g of Safranine dissolved in 100 ml of 70 per cent Ethanol

Staining Apparatus

The equipment consists of a stand (1) with a staining frame on which the microscopic slides are placed and of an electric heating appliance (2 3) for heating of the slides (4) The stand is made of stainless steel It is usually placed over a washing sink The staining frame in the upper part of the stand consists of two parallel edge placed serratured plates (5) of an approximate breadth of 2 5 cm the ends of which are attached to transverse beams (6) which in their turn

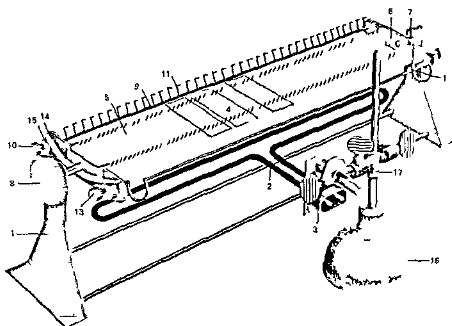


Fig 1

are movably secured to the stand by axles (7) They constitute the support for the microscopic slides and may as occasion requires by means of a wheel (8) be slanted forwards–downwards and in order to wash the back of the slides also be turned round forwards A catch prevents them from being turned backwards In front of the serratured plates there is attached a narrow steel bar (9) with a wheel (10) and curved steel wires (11) intended to intercept the slides when these are slanted more or less forwards for the purpose of washing decolorizing counterstaining etc Behind the plates there is a similar bar (12) with a wheel (13) but without wires This bar is intended to close over the slides while these are still resting on the wires and to keep them in place if it is necessary to wash off staining solution from the back of the slides Both steel bars with their wheels are secured by means of steel springs (14) which rest on eccentric disks (15) coupled to the wheels

The electric heating appliance (2) is provided with an ironing contact (3) for connection to the mains It hangs on a stand (16) with a doublemuff (17) so that it can be adjusted under the preparations in an expedient manner (Fig 1)

Staining Washing Decolorizing and Counterstaining

Preparations on microscopic slides fixed in the usual manner (4) are arranged in a row on the horizontally placed staining frame (a) after the bars on both sides (9 12) have been turned down The Nachtblau solution is carefully dripped onto all the preparations The electric heating appliance (2 3) is placed underneath at a suitable distance from the preparations (approximately 5 cm) The electric current is turned on and the preparations are heated until they form a light vapor This takes approximately 5 min depending on the distance between the heating appliance and the microscopic slides A longer time of heating may occasionally be necessary e g when a tissue section is to be stained The staining solution must not be allowed to evaporate or dry up After the heating the current is turned off and the heating appliance lifted away The preparations are then allowed to cool for a period of 5 min or longer The bar with the curved wires (9) is turned up towards the preparations By means of the large wheel (8) the slides are then turned more or less forwards downwards They are kept in place by the wires The staining solution is allowed to run off the preparations are washed in distilled water and treated for a few seconds with 9 per cent nitric acid Immediately after the preparations are decolorized in 70 per cent ethanol until no more colour comes off i e until the preparations are apparently decolorized After this process washing in water is performed

Counterstaining is carried out with e g pyronine for a few seconds or more when sections are stained If a yellow counter stain is desired the preparations may be treated with chrysoidine solution after the pyronine staining The yellow chrysoidine covers the red pyronine Tissues and non acid fast micro organisms appear more clearly in this case than after counterstaining with chrysoidine alone Afterwards the preparations are blotted with filtering paper and dried Should longer time be required for decolorizing with 70 per cent ethanol or for counter staining which may be necessary e g for the staining of tissue sections the slides are turned upwards to a horizontal position and the wires are if need be turned off because as long as the liquid is in connection with the wires it runs off readily After the necessary period of time the wires are turned on again the preparations are slanted counter stained washed in water and dried

Should some stain apparently be left on the back of the slides the other steel bar (12) may be swung over the slides while they are still resting against the wires and all the preparations turned right round forwards so that the back may be washed clean The slides are then turned upwards again blotted with filter paper and dried

This apparatus was designed for the purpose of staining acid fast bacilli It can also be used for the staining of ordinary more easily stainable micro organisms without heating In that case a simplified

modification of the apparatus may be used in which the curved wires are attached directly onto the foremost serratured edge placed plate (5), so that the preparations when slanted forwards downwards are simply gathered up by the wires

A method of staining several preparations at the same time is described (*Hallberg* 1946) A number of microscopic slides are put into a stain basket and immersed into Nachtblau staining solution which is under heating Such a solution is fully serviceable even after repeated heatings This method has therefore proved practical and time and labour saving especially when many preparations have to be stained daily *The material to be examined must, however be securely fixed to the slides* Pure cultures of tubercle bacilli are difficult to fix Consequently these bacilli should be stained in a special basket or separately *e g* in the apparatus described above

REFERENCE

Hallberg V A new method for staining tubercle bacilli applicable also to the micro organisms of leprosy and other acid fast germs Acta Med Scand Suppl CLXXX p 1–37 1946

Acta path et microbiol scandinav 74 241–258 1968

The Departments of Treponematoses and Biophysics Statens Seruminstitut,
Copenhagen Denmark.

ELECTRON MICROSCOPY OF *TREPONEMA PALLIDUM* NICHOLS

By

OLE B JEPSEN KARI HOVIND HOUGEN and
AKSEL BIRCH ANDERSEN

Received 7 III 68

The morphology of several *Treponema* species has been studied by means of electron microscopy using the techniques of shadow casting, negative staining and ultrathin sectioning (1 2 12 13 14 22 33)

The presence of flagella on these organisms has been the subject of considerable controversy Noguchi (24) believed that the treponemes had an axial filament which was responsible for motility and that it differed from a flagellum only in that it was intracellular The first electron micrographs showed fine thread like appendages which were interpreted as flagella (8 17 18 19 36) Later investigators (25 26 29 34) observed no flagella

In this paper some results of an investigation using ultrathin sectioning and negative staining techniques on *Treponema pallidum* Nichols are presented In particular observations on the number and mode of attachment of the intracellular filaments of this organism as well as on the substructure of these filaments—which was found to be similar to that of bacterial flagella—will be dealt with in detail

MATERIALS AND METHODS

The strain of *Treponema pallidum* Nichols[1] was maintained by intratesticular passage in rabbits 75 – 150 × 10⁶ treponemes were inoculated into each testes and 8–13

Supported by a grant from WHO

The authors wish to thank Mr *F Laursen* Mrs *H Iversen* Mrs *J Berg* and Miss *A C Overgaard* for their excellent technical assistance Our thanks are also due to Dr *H Aa Nielsen* of the Treponematoses Department Statens Seruminstitut for enthusiastic help and criticism

[1] "The Nichols strain was isolated in 1912 by Major *H J Nichols* of the U S Army from the spinal fluid of a patient with recurrent syphilis (see Nichols H A & *Hough W H* Demonstration of S pallida in the cerebrospinal fluid" J Amer med A 60 108 1913) and has been maintained more or less continuously in laboratory animals since that time Periodically one or more accidental laboratory infections of human beings have been observed with this strain testifying to its continued pathogenicity for man despite long propagation in laboratory animals One such instance has in recent years been described from the State Serum Institute Copenhagen (H Aa Nielsen Laboratory infection in man by T pallidum (Nichols)" Document WHO VDT/RES 0 1965) In 1951 the Serological Department of the

days later the animals were killed with chloroform The testes were removed and placed in a 4 per cent solution of formalin which was prepared from paraformaldehyde and adjusted to pH 7 2 with 0 1 N *sodium hydroxide (27) The testis was* split longitudinally and 10–15 incisions towards the sinus were made in each part A fresh portion of the rinsing medium (4 per cent buffered formalin) was taken after this procedure At this stage the testis was placed in 12 ml of the 4 per cent buffered formalin solution and thoroughly shaken for 20 minutes for extraction of the treponemes The extract was pipetted off and centrifuged at room temperature at low speed (1800 r p m in an Ecco superior centrifuge) The supernatant was further centrifuged for 60 minutes at 3600 r p m and the pellet thus obtained was resuspended in redistilled water which had just been filtered through a 0 22 μ millipore filter This suspension was now centrifuged for 10 minutes at 1000 r p m to remove coarser tissue fragments and the supernatant from this procedure was finally centrifuged for 30 minutes at 3600 r p m The pellet of organisms thus obtained constituted the material used for negative staining or for embedding of organisms. In some experiments 3 per cent glutaraldehyde in cacodylate buffer pH 7 2 was substituted for the formalin during the extraction and purification procedure The particular method of fixation and staining is stated in the legend for each figur

Negative Staining

The treponemes *were suspended in redistilled water or in filtered TPI medium (23) to a final concentration of about* 10^9 *organisms/ml* 0 2 ml of this suspension was mixed with an equal volume of stain (3) The stains used were

1) 1 per cent (w/v) ammonium molybdate adjusted to pH 7 with NH_4OH
2) 1 per cent (w/v) phosphotungstic acid adjusted to pH 7 with KOH
3) 1 per cent (w/v) uranyl acetate pH 3 unbuffered

The mixture of organisms and staining material was placed on carbon reinforced Formvar-coated grids A thin film of suspension in a 5 mm platinum loop was applied to the surface of the grid which was placed on filter paper In this way an immediate removal of the excess suspension and a reasonable dispersion and instantaneous drying of the material withheld on the supporting membrane of the grid was achieved (21)

Fixation of Treponema pallidum *for Sectioning*

For preparation of ultrathin sections a suspension of cleaned treponemes from *two or more rabbits* was used A 12 ml sample with a density of about 10^8–10^9 organisms/ml was treated as follows

The suspension was prefixed with 3 per cent glutaraldehyde in veronal acetate buffer pH 6 1 and freed from cellular debris by differential centrifugation as above The pellet after the last centrifugation was enrobed in warm melted agar at 45 C (1 5 per cent Noble Agar Difco in veronal acetate buffer pH 6 1) *Agar blocks of about 1mm³ were cut and fixed overnight at room temperature in 1 per cent* O O$_4$ in veronal buffer pH 6 1 to which *was added* 10 per cent YAP medium (yeast extract —sodium acetate—peptone medium 0 3 0 05 and 0 3 per cent respectively of the Difco products) After a *brief wash in* veronal acetate buffer the blocks were treated for 1 hour with 2 per cent *buffered uranyl acetate pH 6 1 (31) acetone dehydrated* and embedded in Westopal W (32) A few specimens were prepared using glutaraldehyde prefixation in cacodylate buffer 7 2 and post fixation with osmium in veronal acetate at the *same* pH Prefixation with formalin or glutaraldehyde of infected testicular tissue was carried out by *supravital perfusion The rabbit was* anesthetized *with an* ether nitrous oxide gas mixture Aorta abdominalis was exposed immediately distally to the diaphragm and a polyethylene catheter was inserted Vena cava *inferior* was cut wide open simultaneous with the beginning of the in

---

State Serum *Institute acting* as Reference Centre for the World Health Organization received from the International Treponematosis Laboratory Center Department of Microbiology *Johns Hopkins University Baltimore* USA (Professor T B Turner) a rabbit inoculated with the Nichols *strain of T Pallidum The* strain has since been maintained at the State Serum Institute by animal passage"

stillation through the catheter of 3 per cent glutaraldehyde or 4 per cent formalin with or without 0.045 g sucrose/ml added (4). Induration of the testes and hind limbs was apparent within a few minutes and after approximately 10 minutes complete fixation of the hind part of the body including the organs of the abdominal cavity was observed. At the same time the reflux from the vena cava showed only a slight tinge of blood. The animal was now sacrificed while still in deep narcosis and the testes were removed. The testicular tissues were stored overnight at +4 °C in 0.2 M sucrose in cacodylate buffer pH 7.2. Small blocks of about 1 mm³ were cut from both testes and fixed for 1 hour at room temperature in 1 per cent OsO_4 in veronal acetate buffer pH 7.2 with 0.045 g sucrose/ml (4). After a brief wash in buffer with sucrose the blocks were dehydrated in alcohol and propyleneoxide (16) and embedded in Vestopal W.

Sectioning

Ultrathin sections were obtained on the LKB ultratome microtome and post stained with magnesium uranyl acetate (7) and lead citrate (30).

Electron microscopy was carried out on a modified Philips EM 100B and a Philips EM 200 electron microscope at primary magnifications of 1500 × 9000 × 16000 × and 33000 ×. Negatives were obtained on Kodak Fine Grain Safety Positive film and were photographically enlarged as desired. For this paper approximately 1600 recordings have been studied.

RESULTS

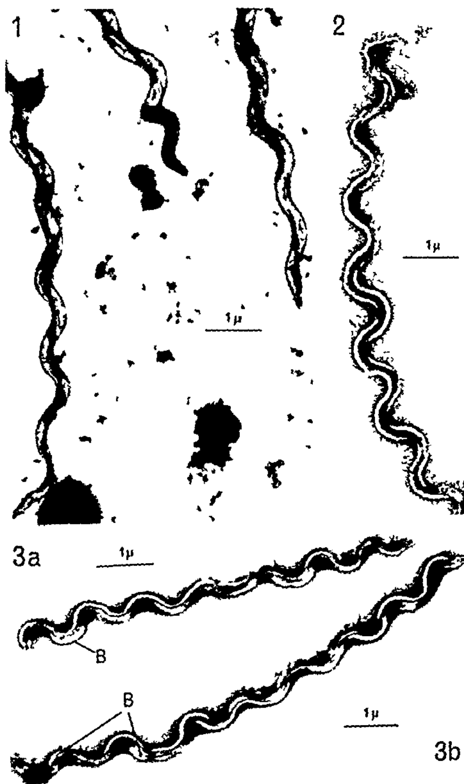
Observations on Negatively Stained Organisms

The length of the treponemes measured along the axis of their helices is about 10–13 μ in average (Figs. 1, 2, 3a, 3b). The shortest and longest observed is 6 and 20 μ respectively. Excluding the pointed ends an average thickness of 0.15 μ is estimated. About 1.5 μ from both ends the width gradually decreases.

The central part of seemingly undamaged organisms shows a wavy outline with a rather constant wavelength of 0.9–1.3 μ and an amplitude of 0.2–0.3 μ. At both ends however the outline appears more smooth and straight (Figs. 1 and 2).

Regions of varying electron densities are observed in the treponemes. This is especially obvious in parts showing definite cytoplasmic evaginations blebs (Fig. 4). In certain organisms some dense round or ring shaped structures are seen (Fig. 5). These may be true inclusions (e.g. polymetaphosphate granules) or just accumulations in indentations on the surface of the organisms of the heavy metal salts used for staining.

The cytoplasmic membrane is seen tautly to envelop the cytoplasm (Figs. 4, 6a, 6b). The membrane itself is particularly well seen about 0.2 μ from the pointed ends as beyond this region no cytoplasm but cell wall material only seems to be present (Figs. 4 and 5). Consequently a certain zonal distribution of varying densities is observed at the tips of the organisms: 1) A region showing a granular substructure beyond the cytoplasmic membrane; this region probably consists of cell wall material only; 2) a zone inside the plasma membrane presenting a rather electron lucent cytoplasm and 3) a more electron dense part of the cytoplasm around the insertion region of the fibrils (Fig. 5).


1
2
1μ
1μ
3a
1μ
B
B
1μ
3b

A bundle of fibrils is visible in the interior of the organisms (Figs 4 5 6a 6b) This bundle seems to originate in the subterminal region (i e corresponding to zone 3 of the cytoplasm as stated above) and is generally seen to extend through the entire length of the treponeme However the number of these intracellular fibrils seems to vary in individual fields of view In the central part of the cell as many as six individual fibrils can be seen (Fig 9) whereas a number varying between three and six is found more terminally located (Figs 5 6a 6b) At the region of insertion at maximum three fibrils are observed (Figs 5 and 6a) Some composite pictures have been made showing a limited number of organisms in their entire length at a reasonably high magnification (ca 100 000 ×) Such montages show six fibrils in the central part of the organisms and only three in the terminal regions In several micrographs the point of insertion for individual fibrils is clearly depicted and three are always present in either end (Figs 5 and 6a) Close to the point of insertion as well as in the middle part of the cell the thickness of each individual fibril is found to be approximately 160 Å but occasionally a few thinner fibrils may be seen in intermediate regions (Fig 5) The bundle of fibrils is tightly twisted around the cytoplasmic helix This coiling pattern however is not completely regular At some points a single or a few fibrils of the bundle can be seen for some distance to pass straight along the surface of the cytoplasmic body (Fig 4) or to bend rather far away from it thus creating a definite interspace between the fibril and the cytoplasmic membrane Half of each individual turn of the fibrillar helix is quite obviously situated alternatingly on the surface of or underneath the cytoplasmic body of the organism (Figs 4 and 5) Nevertheless we have until now been unable to determine whether this bundle constitutes a left or right handed helix

Fibrils outside the cell wall are seen only on damaged treponemes Here they are seen either still possessing their original wavy outline

---

All illustrations show *Treponema pallidum* Nichols
The following abbreviations are used
B = blebs CL = capillary lumen CM = cytoplasmic membrane CW = cell wall F = hook like bend end on F = fibrils H = hook like bend I = inclusion IC = interstitial cell IL = intermediate layer IP = insertion point M = mesosome N = nuclear region R = ribosome

Figs 1–3

Fig 1 Organisms with wavy outline and a rather constant wavelength Note the pointed ends and the fibrils outside the helical body in a partly damaged cell Formalin fixation positive staining 1 per cent uranyl acetate Magnification 14000 ×

Fig 2 Undamaged organism with a well preserved pointed end Formalin fixation negative staining 1 per cent phosphotungstic acid Magnification 14000 ×

Figs 3a and b Short organisms with the characteristic wavy outline Note the straighter ends and blebs B Formalin fixation negative staining 1 per cent ammonium molybdate Magnification 14000 ×

with wavelength and amplitude nearly as in undamaged organisms (Fig 7) or they are found rolled up adjacent to the cytoplasmic body (Fig 8). The fibrils are seen to bend close to their point of insertion, thus forming a small hook or a hook-like bend (10) (Fig 6a). Other micrographs of the region of insertion show this hook-like bend end on (Fig 5). In such fields are pictured round or oval structures with less dense centres of 75–80 Å surrounded by darker, more electron dense zones of about 100 Å in width (Fig 5). A substructure of the fibrils can be observed on certain micrographs showing detached fibrils deposited directly on the supporting membrane of the grid (Fig 12). Four to five longitudinally arranged subfibrils, each consisting of numerous globular subunits with a diameter of about 30 Å are seen (Fig 13). The subfibrils and the pattern of their subunits fit well with the description given by *Lowy & Hanson* (15) for the substructure of bacterial flagella (type A).

In general the cell wall is seen in close apposition to the cytoplasmic membrane or in intimate contact with the intracellular fibrils (Figs 6a, 6b). At the tip of the organism as well as in regions showing blebs it can be found, however, without any direct contact with these structures (Figs 4 and 5).

Observations on Sectioned Organisms

In ultrathin sections only sinusoidal pieces of the cells are included in each section (Figs 14 and 15). The cell wall is seen to tightly envelop the organisms and to consist of a three layered membrane (Figs 14 and 15). The dimension and character of the triple layered membrane are clearly demonstrable as two dark lines with a narrow light gap in between, the total thickness of the membrane being about 90 Å.

In true cross sections of the organisms the fibrils are seen to be located between the three layered cell wall and the three layered cytoplasmic membrane (Fig 16). In some cross sections of the fibrils a substructure of these can be distinguished. The fibrils appear hollow with 5–7 dense granules arranged in a ring around a less dense centre (Fig 16). On longitudinal sections of the treponemes the fibrils are

---

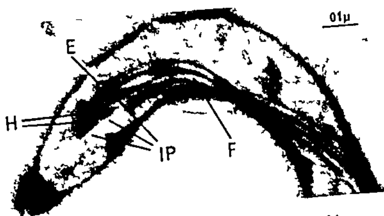
Figs 4–5

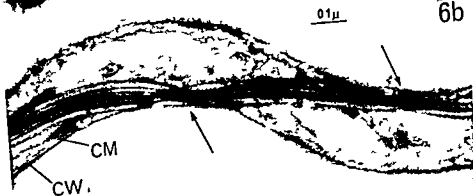
Fig 4 The cytoplasmic membrane (CM) is well seen as a borderline between zones 1 and 2 of the organism (see text). Between CM and CW is cell wall material only (CW). CW is seen in close contact with CM except in the regions of lower electron density, blebs (B) and the part of the cell where the fibrillar bundle (F) passes straight along the cytoplasmic body (arrow). Formalin fixation, negative staining 1 per cent ammonium molybdate. Magnification 90000 ×

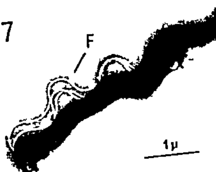
Fig 5 The three zones (see text) at the tip of the organism are clearly illustrated. Three fibrils (F) with insertion points (IP) are seen in zone 3 whereas five fibrils are seen more centrally. Two thin fibrils are present (arrows) and dense inclusions (I) are seen. Formalin fixation, negative staining 1 per cent phosphotungstic acid. Magnification 90 000 ×

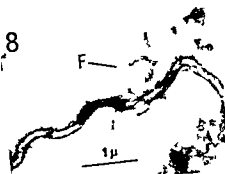

CM
B
CW
0.1μ
F
B
4

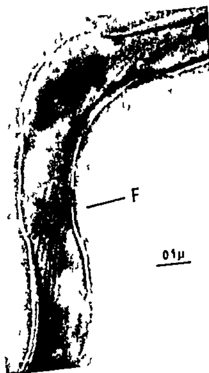

IP
F
0.1μ
F
F
5


0.1μ
6a
E
H
IP
F


0.1μ
6b
CM
CW


7
F
1μ


8
F
1μ


F
0.1μ
9

difficult to see and only in tangentially sectioned parts of some organisms are they observed with a double lined appearance (Fig 15) like bacterial flagella (35) The thickness of the fibrils as seen in ultrathin sections is about 120 Å Until now we have not obtained sections which include the insertion area of the fibrils cut in such a manner that we are able to describe in detail the structures of this region

An amorphous intermediate layer appears to adhere to the outer dark line of the cytoplasmic membrane (Figs 15 and 16)

The cytoplasmic membrane is a well defined three layered structure (Figs 15 and 16) The outer and inner electron dense layers each have a thickness of about 25 Å while the middle layer which is less electron dense has a width of about 30 Å This gives the cytoplasmic membrane an overall thickness of about 80 Å

The cytoplasm contains ribosomes which are rather evenly distributed (Figs 15 and 17) In continuity with the plasma membrane the whorled type of intrusions or mesosomes (6 20) can be seen (Fig 17) The nuclear regions are loose and distinguishable from the cytoplasm as less dense areas containing delicate strands (Fig 17)

The micrographs of ultrathin sections of the infected testes show that the tissue is well preserved (Fig 19) The spaces between interstitial cells however appear swollen (edematous) probably as a result of the infection The treponemes are always seen extracellularly in these spaces They may be found in between the layers of the basal membrane of the tubules but they were never observed inside the tubuli contorti and subsequently have not been seen in the tubular lumen Spermatogenesis seems to be unaffected In the interstitial tissue the treponemes are often seen rather close to capillaries and quite frequently in close proximity to the basal membranes of these (Fig 19) The ultrastructure of the organisms in the infected tissue shows no difference in structure from that observed in the organisms obtained from pellets of extracted cells (Fig 18)

Figs 6–9

Fig 6 a) Three fibrils (F) are inserted at the insertion point (IP) Two fibrils are seen to bend forming a small hook (H) The third shows this hook line bend end on (F) 1) Sharp bends of the fibrillar bundle are present (arrow) Cell wall (CW) is in close apposition to the fibrils and the cytoplasmic membrane (CM) Formalin fixation positive staining 1 per cent uranyl acetate Magnification 90000 ×

Fig 7 A damaged organism showing three fibrils (F) outside the body but still attached at the insertion points The original wavelength of the fibrils is preserved Formalin fixation negative staining 1 per cent ammonium molybdate Magnification 14000 ×

Fig 8 Fibrils (F) rolled up adjacent to the body of a damaged organism Formalin fixation negative staining 1 per cent ammonium molybdate Magnification 14000 ×

Fig 9 Central part of an organism where six fibrils (F) can be seen Formalin fixation negative staining 1 per cent phosphotungstic acid Magnification 90000 ×

DISCUSSION

Preparation of suspensions of *T pallidum* for electron microscopy involves an intensive purification process for removal of testicular tissue fragments Differential centrifugation is generally used for this purpose Either strong centrifugal fields for short periods of time or low fields of gravity for longer periods can be employed In order to minimize the traumatic effects of hard centrifugation a gentle centrifugation technique was chosen Damage by autolysis or other harmful effects of the longer periods employed for centrifugation was prevented by aldehyde fixation prior to and during the extraction procedure Rather low yields of treponemes were obtained per rabbit but the suspensions contained an appropriate number of intact organisms and the amounts of tissue debris were not disturbing A millipore filtration technique (*13*) was found to be unsuitable for the extracts we obtained from rabbit testes

The aldehyde prefixation did not cause any inconvenience for the further handling of the specimens It should be mentioned however that it was difficult to obtain a negative staining of the organisms with uranyl acetate The majority of the cells were positively stained and the micrographs presented in this paper of uranyl acetate treated treponemes all show positive staining

A discrepancy was noticed between the number of intracellular fibrils observed in the subterminal and central regions of the treponemes In several hundred organisms examined three fibrils were found in the subterminal part of the cells and as many as six could be observed in the middle We have been unable to find more than three insertion points for the filaments in the cytoplasm of the subterminal parts of the cells Consequently we are inclined to believe that *T pallidum* Nichols has two sets of fibrils each consisting of three individual fibrils inserted into the cytoplasm at either end of the organism The fibrils are of such a length that a certain degree of overlapping in the central region of the cells is observed Such a polar attachment of

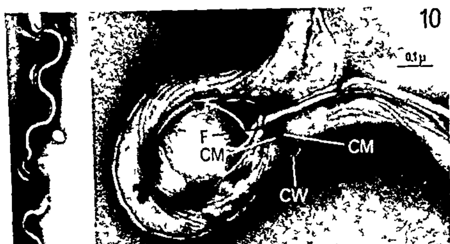
Figs 10 13

Fig 10 Detail of Fig 11 showing division by binary fission The two new organisms are still connected by the cell wall (CW) and fibrils (F) but separated by the cytoplasmic membranes (CM) Magnification 90000 ×

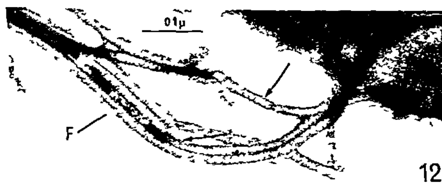
Fig 11 A low power field illustrating the typical Y shaped configuration of a dividing treponeme Formalin fixation negative staining 1 per cent ammonium molybdate Magnification 14000 ×

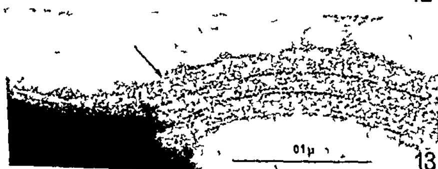
Fig 12 Part of a damaged organism showing fibrils (F) with an indication of substructure Four five rows of subunits can be distinguished (arrows) Formalin fixation staining 1 per cent uranyl acetate followed by 1 per cent ammonium molybdate Magnification 160000 ×

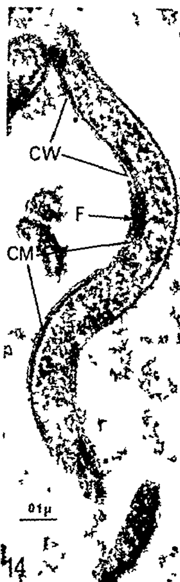
Fig 13 Detail of a damaged organism showing substructure in detached fibrils Five six rows of globular subunits (arrow) are seen Formalin fixation preparation technique as for Fig 12 Magnification 365000 ×

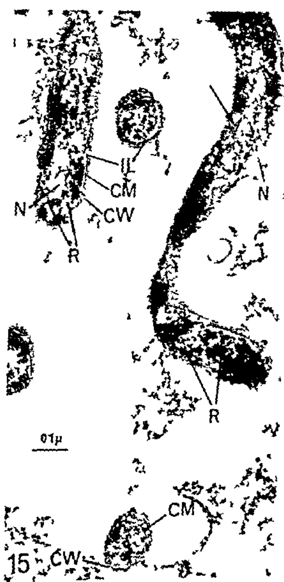

10
0.1μ
F
CM
CM
CW

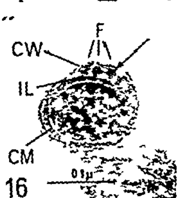

1μ
11

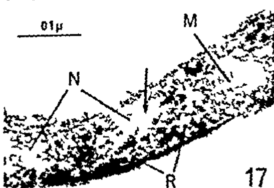

01μ
F
12


01μ
13


CW
CW
F
CM
01μ
14


IL
CM
N
CW
R
N
R
01μ
CM
15
CW


F
CW
IL
CM
01μ
16


01μ
M
N
R
17

fibrils has been observed previously by *Listgarten & Socransky* (14) in a study of some oral spirochetes These authors also emphasize the importance of this type of arrangement for cellular division Later *Pillot* (28) has discussed the possibility that the human pathogenic *T pallidum* also has a similar polar attachment of the fibrils To the best of our knowledge the current literature has no further reports on investigations which can confirm or disprove this theory The present study however strongly indicates 1) that *T pallidum* Nichols does possess two bundles of intracellular fibrils with one bundle attached at either pole 2) that three fibrils are present in each bundle and 3) that each bundle is tightly wound around the cytoplasmic body of the organism In the central region of the cell the individual fibrils of the two groups interdigitate thus forming a single bundle This type of organization of the fibrillar bundle and its implication for the motility of *Treponema microdentium* was discussed in the paper referred to above (14) During drying of the specimens for staining (negative as well as positive) the organisms are subjected to considerable surface tension forces This is clearly seen for instance in the fibrillar bundle The sharp bends present on the individual fibres of the bundle at the circumference of the cell are probably the results of a compression of the entire fibrillar helix during the drying process (Fig 6b) It is also probable that the few fibrils which do not follow the main bundle in some organisms but which seem to have detached parts of the cell wall from the cytoplasmic membrane are artifacts produced during drying

The substructural pattern observed in individual fibrils of the fibrillar helix agrees well with that described by *Lowy & Hanson* (15) for type A bacterial flagella It is thus tempting to consider the intracellular fibrils of *T pallidum* Nichols as being endoflagella This idea is in accordance with the conclusion drawn by *Listgarten & Socransky* (14) in their study of some oral spirochetes and *T microdentium* Further support for the hypothesis of the fibrils being flagella which

---

Figs 14–17

Fig 14 Section showing a sinousoidal piece of an organism The three layered cell wall (CW) tightly envelopes the organism Fibrils (F) are located between CW and the cytoplasmic membrane (CM) (arrow) Magnification 90000 ×

Fig 15 Section showing pieces of organisms cut at various angles The cell wall (CW) is seen outside the intermediate layer (IL) which is adjacent to the outer layer of the cytoplasmic membrane (CM) Ribosomes (R) and nuclear regions (N) are seen in the interior of the cells Note double lined appearance of fibrils (arrow) Magnification 90000 ×

Fig 16 A cross section showing the location of fibrils (F) between the three layered cell wall (CW) and the three layered cytoplasmic membrane (CM) The intermediate layer (IL) is seen to adhere to the outer layer of CM Hollow subfibrils can be distinguished in individual fibrils (arrow) Magnification 160000 ×

Fig 17 A section illustrating the whorled type of mesosome (M) Delicate strands (arrow) are present in the nuclear region (N) Ribosomes (R) are also seen Magnification 160000 ×


C
CM
R
F
C
18


19

are unable to penetrate the cell wall of the treponeme may be found by comparing their insertion into the cytoplasm with that of bacterial flagella. Recently published papers on this subject (9, 11) clearly show the small hooks to end in a basal body within the cytoplasm of *Proteus mirabilis*. A more complex basal organelle consisting of two interconnected basal discs attached to the little hook was observed for individual flagella of the flagellar tuft in *Rhodospirillum* species (5). For references on earlier studies on the attachment of bacterial flagella the reader is referred to these papers. Due to variations in the amounts of heavy metal salts accumulated—probably depending somewhat on the orientation of the organisms—the detailed substructure of the insertion point of the fibrils of *T. pallidum* has not yet been completely resolved. However, the ring shaped structure observed at the end of the hook inside the cytoplasm may very well be the basal organelle or the basal body of the fibril. Negative staining of enzyme treated cells combined with properly oriented sections through the region of insertion will probably provide a basis for further elucidation of the substructure of the terminal parts of the fibrils.

Cell division in *T. pallidum* Nichols occurs by binary fission (Figs. 10 and 11). Until now it has been impossible to establish with certainty whether new filaments are produced or if the already existing filaments also divide by a fission process. So far we have been unable to demonstrate any tails of old filaments as observed for dividing oral spirochetes by *Listgarten & Socransky* (14). Neither has any shedding of filaments in connection with formation of daughter cells been noticed. It must be emphasized, however, that until now only a very limited number of micrographs of dividing cells have been available for study.

Several investigators have found a three layered cell wall in cultivated non pathogenic treponemes (25, 26, 33). The present investigation shows this structure in the cell wall of *T. pallidum* Nichols. Each layer in the cell wall seems to be thicker than the corresponding layer in the three layered cytoplasmic membrane (*e. g.* Figs. 15 and 16).

The cell wall is difficult to resolve when the preparation of organisms for ultrathin sectioning is performed at pH 7.2, while it is clearly distinguished and well defined when the preparation is carried out at pH 6.1. Whether this is due to the difference in pH values or to the different ionic environments is at present unknown.

Figs. 18, 19

Fig. 18. Sectioned organism *in situ* in infected rabbit testes. IC parts of interstitial cell. CM cytoplasmic membrane. F fibrils. R ribosomes. Magnification 90000 ×.

Fig. 19. Section of infected rabbit testes. The treponemes are seen in the space between interstitial cells (arrows) or close to the basal membrane of the capillary. Capillary lumen CL. Magnification 14000 ×.

An electron dense layer is observed between the cell wall and the cytoplasmic membrane (Fig 16) This intermediate layer is seen in intimate contact with the outer part of the cytoplasmic membrane This is in agreement with the schematic drawing of the treponemal membranes presented by *Pillot* in his thesis (28) The membranes surrounding *T pallidum* Nichols are thus in complete accordance with the accepted idea of a cell wall intermediate layer (mucopeptide) cytoplasmic membrane complex of the gram negative bacteria

SUMMARY

The ultrastructure of *Treponema pallidum* Nichols has been investigated by means of ultrathin sectioning and negative staining techniques Some organisms were obtained after an extraction and a purification process for removal of testicular tissue fragments and others were studied *in situ* in infected rabbit testes after prefixation of the tissue by a supravital perfusion technique

The number mode of attachment and the substructure of the intracellular fibrils are described *Treponema pallidum* Nichols has two bundles of filaments each consisting of three individual fibrils inserted into the cytoplasm at either end of the organism The substructural pattern of the individual fibrils agrees well with that of bacterial flagella

Ultrathin sections show the same organization of the membranes surrounding *Treponema pallidum* Nichols as that now generally accepted for gram negative bacteria

The ultrastructure of the organisms in the infected tissue is identical with that observed in organisms extracted from such tissue

REFERENCES

1 *Bladen H A & Hampp E G* Ultrastructure of *Treponema microdentium* and *Borellia vincentii* J Bact 87 1180–1191 1964

2 *Bradfield J R G & Cater D B* Electron microscopic evidence on the structure of spirochetes Nature 169 944 946 1952

3 *Brenner S & Horne R W* A negative staining method for high resolution electron microscopy of viruses Biochim Biophys Acta 34 103 110 1959

4 *Caulfield J B* Effect of varying the vehicle for osmic acid in tissue fixation J biophys biochem Cytol 3 827 830 1957

5 *Cohen Bazire G & London J* Basal organelles of bacterial flagella J Bact 94 458 465 1967

6 *Fitz-James P C* Participation of the cytoplasmic membrane in the growth and spore formation of bacilli J biophys biochem Cytol 8 507 528 1960

7 *Frasca J M & Parks V R* A routine technique for double staining ultrathin sections using uranyl and lead salts J Cell Biol 2? 157–161 1965

8 *Hampp E G Scott D B & Wyckoff R W G* Morphologic characteristics of certain cultured strains of oral spirochetes and *Treponema pallidum* as revealed by the electron microscope J Bact 56 755 769 1948

9 *Hoeniger J F M Heerson W van & Nijman van Zanten F* Basal bodies of bacterial flagella in *Proteus mirabilis* II Electron microscopy of negatively stained material J Cell Biol 31 603 618 1966

10 *Iterson W van* Some remarks on the present state of our knowledge of bacterial flagellation In Bacterial Cytology Symposium 6th International Congress of Microbiology Rome 1953 p 24-38

11 *Iterson W van Hoeniger J F M & Nijman van Zanten E* Basal bodies of bacterial flagella in *Proteus mirabilis* I Electron microscopy of sectioned material J Cell Biol 31 585-601 1966

12 *Kawata T & Inoue T* Fine structure of the Reiter treponeme as revealed by electron microscopy using thin sectioning and negative staining techniques Jap J Microbiol 8 49-65 1964

13 *Listgarten M A Loesche W J & Socransky S S* Morphology of *Treponema microdentium* as revealed by electron microscopy of ultrathin sections J Bact 85 932-939 1963

14 Listgarten M A & Socransky S S Electron microscopy of axial fibrils outer envelope and cell division of certain oral spirochetes J Bact 88 1087-1103 1964

15 *Lowy J & Hanson J* Electron microscope studies of bacterial flagella J molec Biol 11 293-313 1965

16 *Luft J H* Improvements in epoxy resin embedding methods J biophys bio chem Cytol. 9 409-414 1961

17 *Morton H F & Anderson T F* Some morphologic features of the Nichols strain of *Treponema pallidum* as revealed by the electron microscope Amer J Syph 26 565-573 1942

18 *Morton H F Rake G & Rose N R* Electron microscope studies of treponemes III Flagella Amer J Syph 35 503-516 1951

19 *Mudd S Polevitzky K & Anderson T F* Bacterial morphology as shown by the electron microscope V *Treponema pallidum T microdentium* and *T mucrodentium* J Bact 46 15 24 1943

20 *Murray R G E* Fine structure and taxonomy of bacteria In Ainsworth G C & Sneath P H A (Eds) Microbial classification XII Symp Soc Gen Microbiol p 119-144 1962

21 *Murray R G E* On the cell wall structure of *Spirillum serpens* Canad J Microbiol 9 381-392 1963

22 *Wolbert F* Vergleichende elektronenmikroskopische Untersuchungen zur Morphologie von *Treponema pallidum Treponema pertenue* und Reiterspirochaeten Z Hyg Infekt Kr 142 510-515 1956

23 *Niels n H Aa* Investigations on the reproducibility of the quantitative *Treponema pallidum* Immobilization Test Acta path microbiol scand Suppl 40 119-135 19 7

24 Noguchi H The spirochetes In Jordan & Falk (Eds) The newer knowledge of bacteriology and immunity Chicago 1928 p 452-497

25 *Ovčinnikov N M & Delektorskij V V* Anatomy of *Treponema pallidum* WHO/VDT/RES/79 1965

26 *Ovčinnikov N M & Delektorskij V V* Further study of ultrathin sections of *Treponema pallidum* under the electron microscope WHO/VDT/RES/102 1966

27 *Pease D C* In Histological techniques for electron microscopy Academic Press New York and London 1964

28 *Pillot J* Contribution à l'étude du genre Treponema Structures anatomique et antigenique Theses Paris 1965

29 *Pillot J & Ryter A* Structure des spirochetes I Etude des genres Treponema Borrelia et Leptospira au microscope électronique Ann Inst. Pasteur 108 791-804 1965

30 *Reynolds E S* The use of lead citrate at a high pH as an electron opaque stain in electron microscopy J Cell Biol 17 208-212 1963

31 *Ryter A & Kellenberger E* Etude au microscope électronique de plasmas contenant de l'acide désoxyribonucleique I Les nucléoïdes des bactéries en croissance active Z Naturforsch *13b* 597-605 1958

32 *Ryter A & Kellenberger E* L'inclusion au polyester pour l'ultramicrotomie J Ultrastruct Res 2 200-214 1958

33 *Ryter A & Pillot J* Etude au microscope électronique de la structure externe et interne du tréponème Reiter Ann Inst Pasteur *105* 496-501 1963

34 *Swain R H A* Electron microscope studies of the morphology of pathogenic spirochetes J Path Bact *69* 117–128 1955
35 *Tchan Y T & Webber A J* Fine structure and tellurite reduction in *Azotobacter vinelandii* Arch Mikrobiol 54 215–228 1966
36 *Wile U J Picard R G & Kearney E B* The morphology of *Spirochaeta pallida* in the electron microscope J Amer med Ass *119* 880 881 1942

Acta path et microbiol scandinav 74 259 273 1968

Institute for Surgical Research and Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt University Hospital Rikshospitalet Oslo Norway

PHAGOCYTOSIS OF ^{32}P LABELLED *E COLI* BY RAT PERITONEAL POLYMORPHONUCLEAR LEUCOCYTES

Evaluation of a Method

By

ARNE TRIPPESTAD and TORE MIDTVEDT

Received 27 iii 68

The defence of the host against a bacterial invasion is influenced by humoral and cellular mechanisms (1 12) The bactericidal and bacteriolytic properties of mammalian sera against several strains of bacteria have been well documented (1 21 25) Serum factors are also involved in chemotaxis (6 30) and in phagocytosis (6 18) Intracellular degradation of bacteria by macrophages appears also to be dependant on the presence of serum factors (19 24 32)

Bacteria are poorly ingested by phagocytes unless the bacteria have been sensitized by serum factors These factors generally called opsonins can be separated in thermolabile and thermostable components The thermolabile opsonins are probably part of the complement system (6 12 18 29) whereas the thermostable opsonins are more specific and can be removed from serum by absorption with the appropriate antigen (29) The relative participation of the various components may vary with the microbes used (26)

Phagocytosis of bacteria can be assayed *in vitro* either by microscopic examination of stained smears (17) or by methods involving cultivation of nonphagocytosed bacteria from suspensions containing phagocytes and bacteria (13) Microscopic evaluation is difficult especially in smears containing large numbers of bacteria When a strain of bacteria is used which is sensitive to the bactericidal activity of serum results of cultivation methods will not provide reliable information concerning the number of bacteria taken up by phagocytes

During the latter years radio isotope labelling of bacteria has proved to be a useful tool in studies of reticuloendothelial clearance (2 4) It

This investigation was supported by grants from Norsk Medisinaldepot Doktor Alexander Malthes Legat Norwegian Research Council for Science and the Humanities and from the Norwegian Society for Fighting Cancer

Adress Institute for Surgical Research University Hospital Rikshospitalet Oslo Norway

has also been used for studying phagocytosis *in vitro* (9 10 28) The influence of the destructive effect of serum on bacteria in such studies has not however been sufficiently elucidated

According to *Spitznagel & Wilson* (27) the amount of label released to the medium from ^{32}P labelled *E coli* in suspensions containing fresh serum reflects the bactericidal effect of the serum present The release of ^{32}P is apparently caused by damage of the bacterial cell wall by factors in serum which results in loss of cytoplasmic material and bacterial death (5 27)

It has been the purpose of this study to establish an *in vitro* phagocytotic system in which a serum sensitive strain of bacteria could be used The system reported makes it possible to register both rate of phagocytosis and the effect of serum on the bacteria by means of radio isotope technique

MATERIALS AND METHODS

Sodium caseinate (Nutritional Biochemical Co Cleveland Ohio) was dissolved in 0 9 per cent sodium chloride solution to a final concentration of 12 per cent (w/v) The solution was sterilized by autoclaving at 120 C for 30 min and stored at 4 C

Krebs Ringer phosphate buffer with 10 mM glucose was prepared according to *Roberts & Quastel* (23) The abbreviation KRG for this solution will be used in the following

Lowry s alkaline copper solution in this paper called LACS was prepared according to *Oyama & Eagle* (22)

^{32}P was supplied as orthophosphate in a sterile solution containing phosphate buffer by Institutt for Atomenergi Kjeller Norway The specific activity was 0 5 mC per mg of phosphorus

Scintillation fluid was prepared according to *Hall & Cocking* (16) 2 5 diphenyl oxazole (*PPO scint grade Packard Instrument Co Inc La Grange Ill*) 4 g

1 4 bis (2 (4 methyl 5 phenyl oxazolyl)) benzene (dimethyl POPOP scint grade Packard Instrument Co Inc) 0 1 g

Toluene (E Merck AG Darmstadt Germany) 700 ml

2 ethoxyethanol (Fluka AG Buchs SG Switzerland) 300 ml

Formic acid (waterfree Fluka AG Buchs SG Switzerland) 10 ml

All reagents were of analytical grade unless otherwise specified

Glassware

Experiments were performed in tissue culture tubes with a 30 cm² flat bottom Tubes and silicone rubber stoppers were supplied by *Bellco Glass Inc Vineland N J*

Cleaning of Glassware and Stoppers

The tissue culture tubes and stoppers were boiled in soap water for 30 min boiled in tap water for 30 min rinsed in running tap water for 20 hours rinsed in 10 changes of glass distilled water and dried in hot air

Scintillation vials were soaked in soap water for 24 hours at room temperature boiled twice for 30 min in 2 per cent RBS 25 (Chemical Products R Borghgraef Bruxelles Belgium) rinsed for 2 hours in tap water and distilled water in an automatic washing machine and dried in hot air

Sterilization

Glassware was sterilized with dry heat at 160 C for 90 min Stoppers were sterilized in an autoclave at 120 C for 30 min Solutions were sterilized by passage through a Millipore filter of pore size 0 22 μ Antibiotics were not used

Polymorphonuclear Leucocytes (PMN)

were obtained from rats as follows 20 ml of a 12 per cent solution of sodium caseinate was injected intraperitoneally 16–20 hours later the rats were anaesthetized with ether 20 ml of KRG with 10 IU of heparin per ml was injected into the peritoneal cavity After gentle massage the abdomen was opened The fluid was removed with a J shaped pipette and collected in a plastic centrifuge bottle kept in ice water Cells from several rats were pooled The suspension was centrifuged at 200 × g/5 min/0 C in a Sorvall RC2B centrifuge The PMN were washed twice in 100 ml of cold KRG and suspended in the appropriate volume of chilled KRG The number of cells in the suspension was determined in a Bürker counting chamber

Aliquots of 2 5 ml of a cell suspension containing approximately 8 × 10⁶ PMN per ml were incubated in tissue culture tubes at 37 C. After one hour the majority of cells had adhered to the glass surface The medium was removed by decanting and the cell layer was rinsed once with 2 5 ml of ice cold KRG by tilting the tubes gently 10 times

A control tube containing a coverslip was always included. At termination of the experiment the coverslip was removed rinsed in KRG fixed in methanol and stained according to the May Grunewald Giemsa technique

Differential counts of at least 400 random cells revealed that the attached cells consisted of approximately 85 per cent PMN The remaining population consisted mainly of large mononuclear cells among which scattered eosinophilic cells and lymphocytes were found

Serum

Blood was drawn from anaesthetized rats by heart puncture and was allowed to clot at room temperature for one hour It was then kept at 4 C for 3 or 4 hours and centrifuged at 5000 × g/10 min/0 C in a Sorvall RC2B centrifuge Sera from several rats were pooled sterilized through Millipore filter as described and stored at —20 C. The serum was thawed immediately before use Rat serum was used in all experiments

Bacteria

The strain used was isolated from rat faeces and identified as a member of the species *Escherichia coli* according to *Bergey's Manual* (7) The bacteria were stored in lyophilized state at 5 C and were also kept in subculture on lactose bromthymolblue agar plates

Culture Technique

The medium used was prepared according to *Benacerraf et al* ( )

The strain was cultivated in 50 ml of medium for 18 hours From this culture aliquots of 4 ml were used to inoculate 200 ml of medium to which was added 1 mC ³²P labelled orthophosphate The bacterial growth was followed in a Beckman Model C colorimeter at 5 5 mμ When the optical density of the culture reached 0 3 (after approximately 200 min) the growth was stopped by rapid cooling to 0 C At this OD the number of viable bacteria in the culture was estimated to 10⁹ per ml (± 0 5 log) using both tube dilution technique and colony counting on agar plate All incubations were carried out aerobically in a shaker at 37 C

Preparation of the Bacterial Suspension

The bacteria were harvested by centrifugation at 6000 × g/10 min/0 C in a Sorvall RC2B centrifuge The bacteria were washed 3 times with 200 ml of ice cold KRG and suspended in 0 ml of cold KRG From this suspension the appropriate test suspensions used for the experiment were prepared and stored at 0 C The time from the moment the growth was stopped until the bacteria were used in the experiments never exceeded 2 hours

Estimation of the number of viable bacteria revealed that after the manipulations described the number of viable bacteria in the test suspensions was still within the limits indicated *i e* ± 0 5 log The content of radioactivity in the medium was less than 1 per cent of the total radioactivity in the suspension

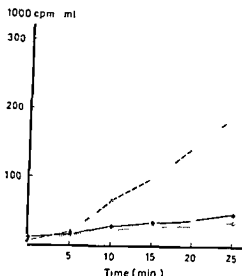


Fig 1

Release of label from bacteria incubated in various media

Aliquots of 2 5 ml of a suspension of ^{32}P labelled *E coli* 10^9 per ml in three different media were incubated at 37 C for various periods of time The media consisted of 1 KRG only (■——■) 2 KRG with 10 per cent serum (Δ----Δ) and 3 KRG with 10 per cent heat treated serum (○ ○) Heat treatment was carried out as follows A glass tube containing 5 ml of the same serum as the one used in the second medium was kept in a water bath at 56 C for 30 min then cooled in ice water The serum was centrifuged at 50000 × g/30 min/0 C in a Sorvall RC2B centrifuge The supernatant was passed through a Millipore filter of pore size 0 22 μ Total radioactivity of the bacterial suspension in this experiment was 791000 cpm per ml Released ^{32}P is plotted as cpm per ml against time of incubation

Phagocytosis Experiments

Unless otherwise stated a suspension was used which contained 10^9 ^{32}P labelled *E coli* per ml in KRG with 10 per cent serum Aliquots of 2 5 ml were added to tissue culture tubes containing rinsed monolayers of PMN The tubes were incubated at 37 C The incubation time was measured from the moment the tube was placed in the incubator until it was removed At termination of incubation the bacterial suspension was poured off and replaced with 2 5 ml of ice cold KRG The cell layer was immediately rinsed in 4 changes of 2 5 ml of cold KRG by tilting the tubes 10 times After drying aliquots of 2 ml of LACS were added to the tubes When the cells were dissolved aliquots of 0 2 ml were removed for determination of radioactivity and cell protein

Release of Label from Bacteria to the Medium

Tubes containing aliquots of 2 5 ml of the test suspension of labelled bacteria but without PMN were incubated at 37 C for various periods of time At the end of incubation the suspension was quickly poured into chilled centrifuge tubes and centrifuged at 6000 × g/10 min/0 C Aliquots of 0 1 ml of the clear supernatant were removed for determination of radioactivity released to the medium This was expressed as cpm per ml

Total Radioactivity in the Bacterial Suspension

To aliquots of 0 1 ml of the bacterial suspension was added 0 1 ml of LACS to dissolve the bacteria Radioactivity was expressed as cpm per ml of suspension

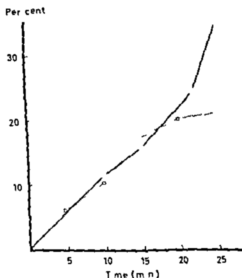


Fig ?

Release of ^{3}P from labelled *E coli* and decrease of bacterial specific activity
Aliquots of 2 5 ml of a suspension of ^{32}P labelled *E coli* 10^{9} per ml in KRG with 10 per cent serum were incubated at 37 C for various periods of time Release of of label to the medium total radioactivity in the suspension and specific activit of the bacteria was determined as described in Materials and Methods
■———■ Decrease in bacterial specific activity expressed as per cent of specific activity at 0 time ○ ○ Release of label expressed as per cent of total radio activity in the ba terial suspension

Sp cific Activity of the Ba teria

Aliquots of 2 5 ml of a su pension of 10^{9} ^{3}P labelled *E coli* in KRG were centri fuged at 6000 × g/10 min/0 C The bacterial pellet was dissolved in 2 ml of LACS Aliquots of 0 2 ml were removed for determination of radioactivity and protein content Specific activity was expressed as cpm per mg of bacterial protein

Determination of Ra lioactivity

Aliquots removed from arious solutions were pipetted directly into scintillation ials to which 10 ml of scintillation fluid was added Radioactivity was determined in a Nuclear Chicago Mark I liquid scintillation counter at —8 C Corrections were ma le f r l ackground counts quenching and radioactive decay

Prote n D termination

Pr tein content of either PMN or bacteria was estimated in aliqu ts of either 0 1 or 0 ? ml of the solution of cells in LACS according to *Oyama & Eagle s* (??) mo li fi ati n of the meth d of *Lowry et al* (20) A solution of lyophilized human albu min (9? per cent purity AB Kabi Sweden) in distille l v ater was used as standard

RESULTS

The illustrations below are based on results from single typical experi ments Individual experiments were carried out two or three times

Preliminary experiments revealed that optimal phagocytosis occurred

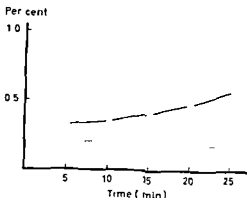


Fig 3

Incorporation into unlabelled *E coli* of ^{32}P labelled inorganic phosphate and of labelled substances released from ^{32}P labelled *E coli*

Unlabelled *E coli* were suspended in KRG with 10 per cent serum 10^9 per ml ^{32}P labelled *inorganic phosphate was added to the suspension 350 000 cpm per ml* Aliquots of 2 5 ml were incubated at 37 C for various periods of time after which total radioactivity in the bacterial pellet was determined as described in Materials and Methods O-----O ^{32}P incorporated in the bacterial pellet expressed as per cent of inorganic ^{32}P labelled phosphate present in the medium ^{32}P labelled *E coli* in KRG with 10 per cent serum 10^9 per ml were incubated at 37 C for 30 min After cooling the suspension to 0 C the bacteria were removed by filtration Un labelled *E coli* were added to the filtrate 10^9 per ml Aliquots of 2 5 ml of this suspension containing unlabelled *E coli* and released labelled substances were incubated at 37 C *for various periods of time Incorporation of label in the bacterial* pellet was determined as described in Materials and Methods ■———■ Radio activity incorporated in the bacteria expressed as per cent of radioactivity in the medium due to released bacterial substances

with 10 per cent serum in the medium For this reason 10 per cent serum was used in all experiments

Exchange of Label between Bacteria and Medium

Release of label to the medium 10^9 ^{32}P labelled *E coli* per ml were selected as the standard number of bacteria in the test suspensions The influence of various factors on the stability of incorporation of label in the bacteria were studied in such suspensions

From Fig 1 it will be apparent that there was considerable release of ^{32}P to the medium when suspensions of ^{32}P labelled *E coli* in KRG with 10 per cent serum were incubated at 37 C The release of label increased with the duration of incubation In KRG alone or in KRG with 10 per cent heat treated serum the rate of release to the medium was small Details are outlined in the figure text

When control suspensions of ^{32}P labelled *E coli* in KRG with 10 per cent serum were kept in ice water bath for two hours only a small amount of label was released to the medium

Suspensions of ^{32}P labelled *E coli* in KRG with 10 per cent serum incubated at 37 C for 15 min *resulted in a considerable release of*

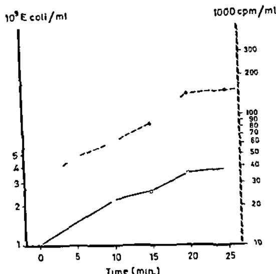


Fig 4

Release of label from bacteria compared to decrease in the number of viable bacteria Aliquots of 2 5 ml of a suspension of ^{32}P labelled *E coli* in KRC with 10 per cent serum 10^9 per ml were incubated at 37 C for various period of time Tubes in triplets were used for each period of incubation From one of the tubes samples were immediately removed for estimation of the number of viable bacteria Both tube dilution technique and colon counting on agar plates were used The two other tubes were chilled to 0 C, and used for determination of released ^{32}P as described in Materials and Methods The number of viable bacteria after various periods of incubation was subtracted from the initial number The difference was taken as the number of bacteria killed at various time intervals The results are plotted on semilogarithmic paper ■------■ Release of ^{32}P per ml ○——○ Number of killed bacteria per ml

labelled material to the medium No increase in release of label was obtained when this suspension was kept at 0 C for further two hours

From Fig 2 it can be observed that during an incubation period up to 20 min at 37 C loss of label to the medium was accompanied by a comparable decrease in specific activity of the labelled bacteria

Reincorporation of ^{32}P into bacteria From Fig 3 it will be apparent that reincorporation into bacteria of either inorganic ^{32}P labelled phosphate or of labelled substances released from ^{32}P labelled *E coli* was insignificant during the incubation period used

Release of label correlated to bacterial viability As illustrated in Fig 4 there was a decrease in the number of viable bacteria during incubation at 37 C of a bacterial suspension containing 10 per cent serum Under the conditions outlined in the figure text the decrease in number of viable bacteria after various periods of incubation paralled release of label to the medium

Influence of various factors on phagocytosis Fig 5 reveals the influence of the composition of the medium on uptake of label by the PMN

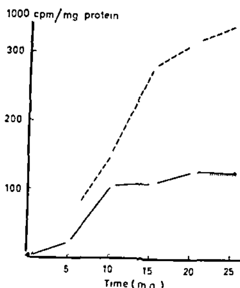


Fig 5

Phagocytosis of ³²P labelled *E coli* suspended in media of different composition ³²P labelled *E coli* 10⁹ per ml were suspended in three different media Aliquots of 2 5 ml of the suspension were incubated at 37 C for various periods of time in tubes containing monolayers of PMN Uptake of radioactivity by the PMN was determined as outlined in Materials and Methods ○——○ Suspending medium KRC △– – –△ Suspending medium KRC with 10 per cent serum ■——■ Suspending medium KRC with 10 per cent heat treated serum Heat treatment was carried out as described in legend to Fig 1

The presence of serum stimulated phagocytosis markedly Heat treated serum did not show the same stimulating effect

It will be seen from Fig 6 that under the conditions described in the figure text there was an almost linear correlation between uptake of label by the PMN and the number of bacteria in the test suspensions up to 10⁹ bacteria per ml

The influence of incubation time on uptake of ³²P by the PMN will be apparent from Figs 5 7 and 8 Nearly maximal phagocytosis occurred after 15 to 20 min incubation at 37 C

Exchange of Label between Phagocytes and Medium

Incorporation of ³²P not bound to bacteria Incorporation into PMN of inorganic ³²P labelled phosphate and of ³²P labelled substances released from labelled bacteria was determined From Fig 7 it will be seen that incorporation of either was rather small *i e* less than 2 per cent of the total radioactivity taken up by the PMN during phagocytosis of labelled *E coli* Experimental conditions are described in the figure text

Release of label from the phagocytes Release of label from phagocytosed bacteria disintegrating inside the PMN is *demonstrated in Fig* 8 As outlined in the figure text it could be estimated that during an

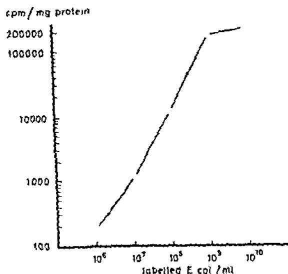


Fig 6

Influence of number of bacteria on the degree of phagocytosis

Aliquots of 2 5 ml of a logarithmic dilution of a suspension of ^{32}P labelled *E coli* in KRG with 10 per cent serum were incubated at 37 C in tubes containing monolayers of PMN A standard incubation time of 15 min was used Uptake of ^{32}P by the PMN was determined as outlined in Materials and Methods Uptake in cpm per mg of cell protein is plotted on semilogarithmic paper against the number of bacteria per ml of suspension

incubation period up to 20 min release of label to the medium never exceeded 10 per cent of the ^{32}P taken up by the PMN

Reproducibility of results A suspension of ^{32}P labelled *E coli* 10^9 per ml in KRG with 10 per cent serum was prepared Aliquots of 2 5 ml were added to tubes containing monolayers of PMN and also to control tubes without PMN The PMN were pooled from 3 rats A standard incubation period of 15 min at 37 C was used Release of label to the medium and phagocytosis was determined as described in Materials and Methods Dispersion of data from such an experiment is presented in Table 1

DISCUSSION

Two different *in vitro* systems are commonly used for the determination of phagocytosis Phagocytes and bacteria can be kept in suspension during the experimental period (9 13 14 18) or the experiments can be carried out with phagocytes adherent to a surface (8 15 29 31 32)

The latter system has certain technical advantages Washing of the cell layer before adding the bacteria will remove unwanted cells like erythrocytes lymphocytes and damaged phagocytes Furthermore the

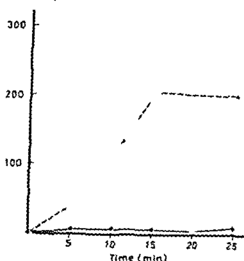


Fig 7

Incorporation into phagocytes of ^{32}*P labelled inorganic phosphate and of labelled substances released from* ^{32}*P labelled E coli*

To a suspension of unlabelled *E coli* in KRG with 10 per cent serum 10^9 per ml ^{32}P labelled inorganic phosphate was added 350 000 cpm per ml Aliquots of 2 5 ml of this suspension were incubated at 37 C in tubes containing monolayers of PMN After various periods of incubation uptake of ^{32}P by the PMN was determined as described in Materials and Methods (○———○) ^{32}P labelled *E coli* 10^9 per ml suspended in KRG with 10 per cent serum were incubated at 37 C for 30 min After cooling the suspension to 0 C the bacteria were removed from the medium by filtration The same number of unlabelled *E coli* suspended in KRG with 10 per cent serum was also incubated at 37 C for 30 min cooled to 0 C and collected by centrifugation at 6000 × g/10 min/0 C in Sorvall RC2B centrifuge The unlabelled preopsonized *E coli* were suspended in the filtered medium which contained released labelled substances Aliquots of 2 5 ml of the suspension thus obtained were incubated at 37 C for various periods of time in tubes containing monolayers of PMN Incorporation of such substances in the PMN was determined as described in Materials and Methods (■———■) As control aliquots of 2 5 ml of a suspension of ^{32}P labelled *E coli* 10^9 per ml in KRG with 10 per cent serum were incubated at 37 C for various periods of time in tubes containing monolayers of PMN Phagocytosis of ^{32}P labelled bacteria by the PMN was determined as outlined in Materials and Methods (△– – –△)

problem of separating extracellular bacteria from the phagocytes is easily solved by using cells adherent to a surface (8, [illegible]) For these reasons the latter system was used in this study The cell population in our experiments consisted of approximately 85 per cent PMN the remaining were primarily large mononuclear cells

The use of viable bacteria as test particles warrants exact standardization of procedures In our experiments a fresh culture of bacteria was always used the bacteria being in the same log phase of growth Only a limited alteration in the number of viable bacteria was observed during the manipulations necessary to prepare the test suspensions provided the bacteria were kept at 0 C during the period of preparation

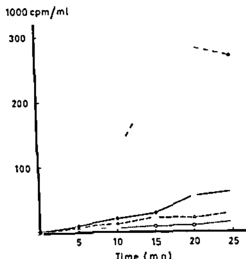


Fig 8

Release of ³P from PMN after phagocytosis of labelled *E coli*

Aliquots of 2 5 ml of a suspension of ³P labelled *E coli* 10⁹ per ml in KRG with 10 per cent serum were incubated at 37 C in tubes containing monolayers of PMN After various periods of incubation the cell layers were rapidly washed in 4 changes of KRG Fresh medium containing the same number of unlabelled *E coli* was added and the tubes were again incubated at 37 C Radioactivity was determined in 0 1 ml aliquots of the suspension at 0 10 20 and 40 min as described in Materials and Methods After 40 min the suspension was removed the cell protein of the PMN determined as described The control graph was constructed from values obtained by summation of radioactivity released from and remaining in the PMN (● — ●) The other graphs illustrates released radioactivity after incubation periods of 10 min (○ ○) 20 min (△-----△) and 40 min (■———■)

The strain of *E coli* used was sensitive to the bacteriolytic and bactericidal activity of fresh serum Lysis could not be detected photometrically until the suspension had been incubated at 37 C for more than 60 min On the other hand release of ³P to the medium was apparent already after a few minutes at 37 C About 25 per cent of the radioactivity bound to the bacteria was released to the medium during an incubation period of 25 min This phenomenon makes it desirable to use short incubation periods for studies of phagocytosis with such a strain of bacteria Our results confirmed those obtained by *Spitznagel & Wilson* (27) that there is good correlation between the rate of release of ^{32}P to the medium and bacterial death

Cooling the suspensions will effectively stop the serum dependent release of ^{32}P from labelled *E coli* (27) Our results confirmed this finding

It is desirable in such studies to use *E coli* in numbers large enough to yield high radioactivity in the samples This will minimize counting errors By the technique used 10⁹ bacteria per ml of medium was found suitable

TABLE 1

Precision of Methods for Determination of Phagocytosis and Release of Label from Bacteria

No	Phagocytosis cpm/mg protein	Release cpm/ml
1	124856	89740
2	120403	86460
3	132628	93600
4	122314	84620
5	104494	103910
6	124697	115190
7	121177	116780
8	134099	93860
9	110516	91600
10	118164	100120
11	147046	95480
12	119107	84780
13	124183	83250
14	122274	123580
15	122250	77640
16	123391	72390
17	116116	92070
18	122757	78940
19	124477	90300
Mean	122923	93332
S D	8817	13580
S E M	2022	3115

S D standard deviation S E M *standard error of the mean*

Several sources of error should be evaluated before the system described can be deemed useful for practical purposes The major source of error is due to loss of label from the bacteria Within an incubation period of 20 min loss of label is accompanied by a decrease in specific activity Apparently ^{32}P labelled substances are lost earlier than bacterial protein provided that there is no increase in protein content of the bacteria Consequently the actual uptake of bacteria by the PMN will be higher than that indicated by the measured incorporation of label This source of error will not however exceed 20 per cent during an incubation period of 15 min calculated from the amount of ^{32}P lost from the bacteria With other sera and other bacteria this figure might be different

Reincorporation of label into bacteria is too small to influence evaluation of the data Less than 1 per cent of the radioactivity present in the medium was incorporated in the bacteria during the incubation period used

...ination of cell pr... the end of experiment will include ...in content of the... that of the phagocytosed bacteria ...ill also lead to ... of underestimation of the actual ...PMN From... activity of the labelled bacteria

it can however be calculated that bacterial protein usually constitutes less than 7 per cent of the measured amount

Incorporation of ^{32}P from other sources than intact bacteria cannot constitute a major source of error It has been shown that incorporation of ^{32}P is larger in phagocytosing cells than in resting ones (3) For this reason unlabelled *E coli* were added to the medium during the incorporation studies Our results indicate that less than 2 per cent of the ^{32}P taken up could be explained by incorporation of labelled substances released from bacteria

Expulsion of labelled products from bacteria disintegrating within the phagocytes (11 28) might also be a source of error Results from studies of the rate of expulsion of label indicate that the highest possible release of ^{32}P from the PMN within a 20 min period of incubation could not constitute more than 10 per cent of that taken up by the cells

Due to qualitative differences as well as to variations in the numbers of bacteria and PMN from one experiment to another comparisons should be made between groups in the same experiment Sera from different rats vary in their ^{32}P releasing and phagocytosis stimulating capacity When the effects of various sera were compared there was always parallelity between release of label and the degree of phagocytosis stimulation This suggests that release of label from bacteria has something to do with the process of opsonization

SUMMARY

A method for *in vitro* determination of phagocytosis of ^{32}P labelled *E coli* by rat peritoneal polymorphonuclear leucocytes is described Optimal phagocytosis was found to be dependent on opsonization with heat labile serum components The presence of serum caused a considerable loss of labelled compounds from bacteria to medium which was the main source of error in the system used The influence of other forms of isotope exchange was also evaluated With due consideration of the various sources of error the system described should be a useful tool for the evaluation of aspects of host defence mechanisms

REFERENCES

1 *Austen K F & Cohn Z A* Contribution of serum and cellular factors in host defense reactions I Serum factors in host resistance New Eng J Med 268 933 938 1963

2 *Benacerraf B Sebestyen M M & Schlossman S* A quantitative study of the kinetics of blood clearance of ^{32}P labelled *Escherichia coli* and staphylococci by the reticuloendothelial system J Exp Med *110* 27–48 1959

3 *Borel R R & Karnovsky M L* Biochemical basis of phagocytosis V Effect of phagocytosis on cellular uptake of extracellular fluid and on the intracellular pool of L-a gl cerophosphate Fed Proc 25 840–845 1966

4 *Biozzi G Howard J C Halpern B N Stiffel C & Mouton D* The kinetics of blood clearance of isotopically labelled *Salmonella enteritidis* by the reticuloendothelial system in mice Immunology 3 74–89 1960

5 *Bladen H A Evans R T & Mergenhagen S E* Lesions in Escherichia coli membranes after action of antibody and complement J Bact *91* 2377 2381 1966

6 *Boyden S V North R J & Faulkner S M* Complement and the activity of phagocytes in Ciba Foundation Symposium Complement J & A Churchill Ltd London 190–221 1965

7 *Breed R S Murray E D G & Smith N R* Bergey s Manual of Determinative Bacteriology 7th ed Williams & Wilkins Co Baltimore 1957

8 *Brumfitt W Glynn A A & Percival A* Factors influencing the phagocytosis of *Escherichia coli* Brit J Exp Path *46* 215–226 1965

9 *Br uchowska W* Use of radioactive isotopes in studies on phagocytosis in vitro Nature (London) *212* 210–211 1966

10 *Carpenter R R* Phagocytosis by *guinea pig splenic cells of Escherichia coli* and protein coated bentonite particles labeled with iodine 125 J Immun. *96* 992–999 1966

11 *Cohn Z A* The fate of bacteria within phagocytic cells I The degradation of *isotopically labeled* bacteria by polymorphonuclear leucocytes and macro phages J Exp Med *117* 27–42 1963

12 *Cohn Z A & Austen K F* Contribution of serum and cellular factors in host defense reactions II Cellular factors in host resistance New Eng J Med *268* 1056–1064 1963

13 *Cohn Z A & Morse S I* Interactions between rabbit polymorphonuclear leucocytes and staphylococci J Exp Med *110* 419–443 1959

14 *Cohn Z A & Morse S I* Functional and metabolic *properties of polymorpho* nuclear leucocytes I Observations on the requirements and consequences of particle ingestion J Exp Med *111* 667–687 1960

15 *Craig C P & Suter E* Extracellular factors influencing staphylocidal capacity *of human polymorphonuclear leukocytes J Immun 97* 287 296 1966

16 *Hall T C & Cocking E C* High efficiency liquid scintillation counting of 14C labelled material in aqueous solution and determination of specific activity of labelled proteins *Biochem J 96* 626–633 1965

17 *Hanks J H* Quantitative aspects of phagocytosis as influenced by the numbers of bacteria and leucocytes J Immun *38* 159 176 1940

18 *Hirsch J G & Strauss B* Studies on heat labile opsonin in rabbit serum J Immun *92* 145 154 1964

19 *Jenkin C R* The effect of opsonins in the intracellular survival of bacteria Brit J Exp Path *44* 47–57 1963

20 *Lowry O H Rosebrough N J Farr A L & Randall R J* Protein measure ment with the Folin phenol reagent J Biol Chem *193* 265 275 1951

21 *Muschel L H & Treffers H P* Quantitative studies on the bactericidal actions of serum and complement II Some implications from the mechanism of the bactericidal reaction J Immun *76* 11–19 1956

22 *Oyama V I & Eagle H* *Measurement of cell growth in tissue culture with a* phenol reagent (Folin Ciocalteau) Proc Soc Exp Biol Med *91* 305–307 1956

23 *Roberts J & Quastel J H* Particle uptake by polymorphonuclear leucocytes and Ehrlich ascites carcinoma cells *Biochem J 89 150 156 1963*

24 *Rowley D* Bactericidal activity of macrophages in vitro against *Escherichia coli* Nature (London) *181* 1738 1739 1958

25 *Rowley D & Wardlaw A C* Lysis *of Gram negative* bacteria by serum J Gen Microbiol *18* 529 533 1958

26 *Slopek S Grzybek Hryncewicz K Lalos J & Kubis K* Opsonizing factors in rat serum II Serum component taking part in the phagocytosis of *S typhimurium* Arch Immun Ther Exp *13* 312 317 1965

27 *Spitznagel J K & Wilson L A* Normal serum cytotoxicity for 32P labelled smooth Enterobacteriaceae I Loss of label death and ultrastructural damage J Bact *91* 393 400 1966

28 *Thorpe B D & Marcus S Comparison of two techniques to study in vitro* uptake and fate of *Pasteurella tularensis* J Reticuloendothel Soc *1* 418–422 1964

29 *Vaughan R B* The *discriminative* behaviour of rabbit phagocytes Brit J Exp Path *46* 71 81 1965

30 *Ward P A Cochrane C G & Müller Eberhard H J* The role of serum complement in chemotaxis of leukocytes in vitro J Exp Med 122 327-346 1965

31 *Whitby J L & Rowley D* The role of macrophages in the elimination of bacteria from the mouse peritoneum Brit J Exp Path 40 358-370 1959

32 *Wu W G & Marcus S* Humoral factors in cellular resistance II The role of complement and properdin in phagocytosis and cytopepsis by normal and immune macrophages J Immun 92 397-403 1964

Acta path et microbiol scandinav 74 274 286 1968

The National Bacteriological Laboratory Stockholm Sweden

INVESTIGATIONS ON THE ENZYMES AND TOXINS OF STAPHYLOCOCCI

Relationship of pH to Growth and Production of Enzymes

By

M O TIRUNARAYANAN and H LUNDBECK

Received 17 ii 68

Virulent strains of staphylococci are known to produce a number of enzymes and toxins in large quantities in laboratory culture (3 8) To what extent such enzymes and toxins are formed within host tissues during infection is not established and the roles played by the different components in infection and immunity are still obscure

A number of conditions affect the production of the different extra cellular products of staphylococci in laboratory culture What is optimal for the production of one enzyme is inhibitory to the production of another The availability of certain nutritional factors in the medium is of importance It was earlier shown that lack of glucose or an acid pH condition of the medium diminished the production of coagulase (2 20) Slight alterations in the temperature of growth were also found to affect the production of staphylokinase and hyaluronidase (2) Further the toxic or stimulatory effect of metal ions on the production of enzymes is well established Proper amounts of carbon dioxide and oxygen are important for the production of α haemolysin (11) In order to outline the optimal conditions for the production of the individual enzymes a systematic study of the effect of pH on growth and on the production of extracellular substances was undertaken The results are reported in the present communication

MATERIAL AND METHODS

Strains Staphylococcus aureus strain Wood 46 (variant no 10344 obtained from the National Collection of Type Cultures England) and a laboratory strain "Walker" (phage type 42D) were used The bacteria were stored in the lyophilized state and subcultured in nutrient broth and streaked on nutrient agar plates

Media and solutions Nutrient broth (20) was used for the growing of the Walker strain whereas a casein hydrolysate medium containing inorganic salts and yeast dialysate (20) was used for the cultivation of the Wood strain Glucose when added to the medium was sterilized separately in the autoclave as a ten per cent solution in distilled water Solutions of 30 per cent $KHCO_3$ and K_2CO_3 in distilled water were sterilized in the autoclave whereas 1N NaOH and 1N HCl were made by diluting concentrated solutions in sterile distilled water

Determination of enzymes The culture supernatants used for the determination of various activities were first freed of cellular material by centrifugation at 30000 g for 60 minutes at 4 C. 0 02 per cent sodium merthiolate was added to the super natants to prevent bacterial growth The pH optima for the production of enzymes were different from the pH optima for enzyme activity Therefore the super natants were adjusted to suit the conditions of enzyme assays

Lipase was determined by the egg yolk agar (12) and Tween 60 agar plate methods (21) as well as by titration of the acid formed from tributyrin under nitrogen atmosphere (22) *Coagulase* was measured by determining the plasma clotting activity of serial dilutions of the supernatants (20) Coagulase activity was also determined using suspensions of the centrifuged bacteria in physiological saline containing 0 2 per cent peptone 0 1 ml of the suspensions were mixed with 0 4 ml of rabbit plasma and examined after 2 hours incubation at 37 C. *Protease* was assayed using gelatin as the substrate by the viscosimetric method described by *Tirunarayanan & Lundblad* (24) Similar viscosimetric methods of enzyme assays were used for the determination of *hyaluronidase* (25) and *nuclease* (23) *Phosphatase* was determined by the method of Inniss & San Clemente (10) using p nitro phenyl phosphate as the substrate

Optical density The optical density of bacterial growth was measured at 600 mμ using a Bausch & Lomb Spectronic 20 colorimeter Absorption of solutions from chromatographic experiments were determined at 260 and 280 mμ with 10 mm light path cells and using a Beckman spectrophotometer model DU

Viable counts Determination of viable counts were made by plating 0 1 ml of ten fold dilutions in physiological saline on duplicate nutrient agar plates The plating was done immediately after each step in the dilution series The number of colonies were counted after 48 hours incubation at 37 C. Only plates giving more than 20 and less than 500 colonies were counted

Chromatography on Sephadex G 100 The culture supernatants were concentrated fifty times by ultrafiltration at 5 C using Barkefeld filters coated with 10 per cent *collodion* 5 ml of the concentrate was applied on a column of Sephadex G 100 with a bed size of 2 4 cm diameter and 144 cm length using 0 05 *I* tris HCl buffer of pH 7 4 8 ml fractions of the effluent were collected in an automatic fraction collector

EXPERIMENTAL

Among staphylococcal strains variations in the ability to produce different extracellular substances are considerable Because of this property different strains have to be used when the effect of pH on the production of enzymes is to be studied Apart from this other factors like the composition of the medium the rates of agitation and passage of gas mixtures the time of growth as well as the type of titrant used to maintain pH constant exert a pronounced effect For instance the addition of glucose was found to enhance the production of coagulase (20) whereas it was observed in the present study that glucose inhibited the formation of protease Preliminary studies also showed that nutrient broth which was conducive to coagulase production was not as effective as casein hydrolysate medium for the production of protease High yields of phosphatase were obtained when $KHCO_3$ but not when NaOH was used as the titrant by which to maintain pH constant Thus the use of different strains and different conditions for cultivation cannot be avoided when a comparative analysis of the effect of pH on the production of different enzymes is to be made Two strains Walker and Wood were used in the present study and the production of six different enzymes was investigated The

Walker strain was grown in nutrient broth containing glucose to study the production of coagulase hyaluronidase and phosphatase. The Wood strain was grown in casein hydrolysate medium without glucose to study the production of protease lipase and nuclease.

Maintenance of Constant pH

The equipment used for the cultivation of the bacteria consisted of a two litre glass tank provided with a lid with one central and three eccentric openings. A combined glass electrode was introduced into the culture medium through the central opening. The electrode was sterilized each time with tincture of iodine, washed with ethanol and filled with a sterile saturated solution of potassium chloride and then plugged with sterile cotton. This was done in order to ascertain that bacteria from the previous cultivation did not enter the culture through the porous pin of the electrode. Such a sterilization process did not affect the sensitivity or response of the electrode as tested with different standard buffer solutions.

The three outer openings were intended for the passage of air, for sampling and for the supply of titrant through a magnetic valve regulated automatically by a Radiometer pH titrator. The tank was placed within a bigger vessel which functioned as a jacket and through which water at 37° C was circulated from a thermostated water bath. The medium was stirred by a magnetic stirrer set at 200 r.p.m. The pH values were recorded continuously using an Elmes 12 recorder.

1400 ml of the culture medium, after adjustment of the pH to the set value on the titrator, was inoculated with 1.4 ml of an 18 hour culture in the same medium. When glucose is added, the bacteria liberate acid which shifts the pH value gradually to the acid side reaching a final value around pH 3.9 in 24 hours. By titrating the acid so liberated with an alkali the pH could be maintained constant at any set value between pH 9.0 and pH 4.5 until all the available glucose and its metabolites were oxidized to acid. With one per cent glucose the pH value could thus be maintained constant by titration for a period of up to 48–72 hours.

In preliminary experiments air was passed over the surface of the medium in the tank. Oxidation of glucose, as judged by acid production, was rapid. However such acid production was also found to occur when the rubber stoppers of the two outer openings were replaced by cotton plugs and no air was passed through the tank. The experiments were therefore performed without air passage. As titrants 30 per cent $KHCO_3$ and K_2CO_3 were used. The acid formed from glucose reacted with the titrant and produced carbon dioxide. In experiments without glucose the titrants used were 1N HCl and 1N NaOH.

Effect of pH on the Growth of the Bacteria

The results of experiments on the growth and amount of titrants recorded for the maintenance of pH at constant values are shown in Tables 1 and 2. The Walker strain showed good growth in nutrient broth *containing glucose between pH values of 5.0 and 9.0. The amount* of titrant used up increased gradually as the pH was raised from 4.5 to 8.5. The experiments with the Wood strain grown in casein hydrolysate medium without glucose indicated that growth in the absence of glucose was not as vigorous as when glucose was added (Table 2). For example at pH 6.5 *the number* of viable cells were about 5 10 times higher in the presence of glucose than in its absence. *The optical density of the cultures* also showed a great increase. In the absence of glucose good growth occurred at pH values between 5.0 and 8.0. Growth was considerably inhibited at pH 8.5 and above.

TABLE 1

Effect of pH on Growth of Walker Strain and Amount of Titrant Used for pH Maintenance

pH of growth	Viable count/ml (× 10⁶)		Optical density 600 mμ	Titrant	
	0	24 hours		3.0 c	ml
4.0	0.640	0.95	0	$KHCO_3$	0
4.5	0.155	1.17	0.210	$KHCO_3$	10
5.0	0.603	280	0.830	$KHCO_3$	17
5.5	0.421	2400	1.400	$KHCO_3$	50
6.0	0.771	964	1.360	$KHCO_3$	50
6.5	0.611	458	1.200	$KHCO_3$	50
7.0	0.830	434	1.000	$KHCO_3$	63
7.5	0.563	1280	1.100	$KHCO_3$	120
8.0	0.510	1450	1.100	$KHCO_3$	225
8.5	0.554	601	0.980	$KHCO_3$	420
9.0	0.361	130	0.760	K_2CO_3	73
9.5	0.421	0.013	0	K_2CO_3	0

TABLE 2

Effect of pH on Growth of Wood Strain and the Amount of Titrant Used for pH Maintenance

pH of growth	Viable count/ml (× 10⁶)				Opt density 600 mμ			Titrant	ml used		
	0	24 h	48 h	72 h	24 h	48 h	72 h	1 M	24 h	48 h	72 h
5.0	0.133	101	682	230	0.365	0.640	0.760	HCl	3	7	10
5.5	0.141	133	18	212	0.455	0.660	0.750	HCl	3	7	9.6
6.0	0.220	261	178	159	0.520	0.800	0.730	HCl	3	7	10
6.5	0.128	140	598	294	0.500	0.675	0.700	HCl	0	4	8
7.0	0.170	395	873	410	0.410	0.650	0.710	HCl	0	2	6
7.5	0.143	489	627	356	0.480	0.680	0.750	NaOH	5	6	8
8.0	0.243	140	290	230	0.540	0.690	0.750	NaOH	7	12	22
8.5	0.165	50	64	23	0.380	0.490	0.540	NaOH	8	15	23
9.0	0.156	8	20	12	0.220	0.340	0.310	NaOH	9	17	20
6.5*	0.6[illegible]5	2690	3120	5530	2.00	2.20	4.02	NaOH	110	112	112

* Culture medium contained 1 per cent glucose

Production of Extracellular Substances at Various pH Values

Coagulase Extracellular coagulase was produced by the Walker strain in large amounts in the presence of glucose but not in its absence The effect of pH of growth on the production of coagulase is shown in Fig 1 The bacteria liberated coagulase when grown at pH above 7.0 Maximal coagulase titres were obtained at pH between 7.5 and 8.0 Higher pH gave decreased titre Coagulase activity was tested with the centrifuged bacteria No coagulase activity was demonstrable when the bacteria were grown at pH below 6.5

Phosphatase The relationship between pH of cultivation and the production of phosphatase is shown in Fig 2 Phosphatase was formed

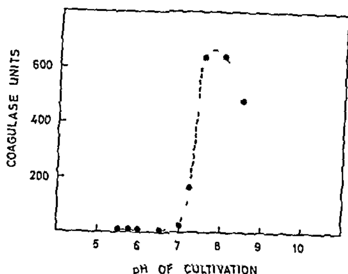


Fig 1

Production of coagulase by strain Walker at various pH

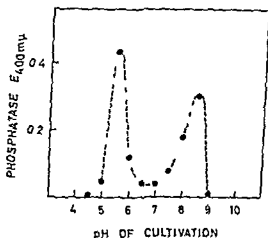


Fig 2

Production of phosphatase by strain Walker at various pH

at two pH regions one between pH 5.0 and 6.0 and another between pH 7.5 and 8.5 The enzyme formed at pH 8.5 appeared early during growth in the medium The small amount of this enzyme produced at the end of four hours incubation gave a pH activity curve with an optimum around pH 6.9 (Fig 3) This agreed closely with the description of the phosphatase studied by *San Clemente & Zolli* (18) However at the end of 24 hours incubation the pH activity curve of the phosphatase produced at pH 8.5 showed a broader optimum with a plateau between pH 4.9 and 6.5 (Fig 4) A similar curve was obtained for the phosphatase produced by the bacteria grown at pH 5.5 giving a peak

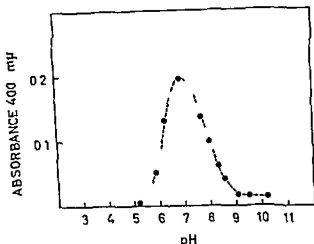


Fig 3
pH activity relationship of the phosphatase formed during the early phase of growth at pH 8 5

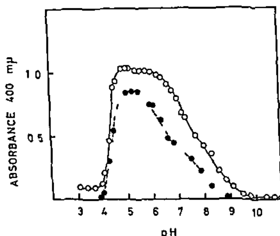


Fig 4
pH activity relationship of the phosphatases produced after 24 hours growth at pH 8 5 (○——○) and at pH 5 5 (●——●)

activity around pH 5 25 (Fig 4) These curves resembled more the phosphatase described by *Barnes & Morris* (1) These results indicated that the Walker strain produced two phosphatases one having an optimum at pH 6 9 and another at pH 5 25 Repeated trials to produce the enzyme component which showed peak activity at pH 6 9 and was free of activity at pH 5 25 were unsuccessful

Experiments done with a view to separating the two activities by chromatography on Sephadex G 100 gel showed that the enzymes oc

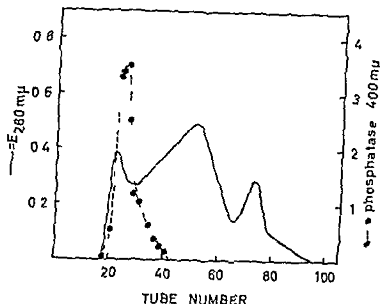


Fig 5

Gelfiltration on Sephadex G 100 of the phosphatase produced by strain Walker at pH 8 5

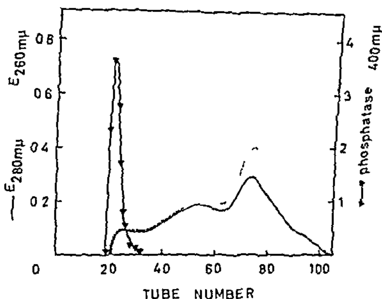


Fig 6

Gelfiltration on Sephadex G 100 of the phosphatase produced by strain Walker at pH 5 5

curred together in a single peak close to the void volume (Figs 5 and 6) Further work is needed to see whether the two activities may be separated by other means It should be noted that the enzyme produced at pH 5 5 and separated on Sephadex G 100 gel had a low con

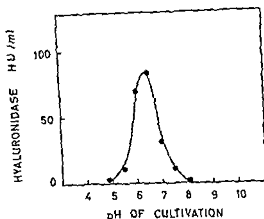


Fig 7
Production of hyaluronidase by strain Walker at various pH

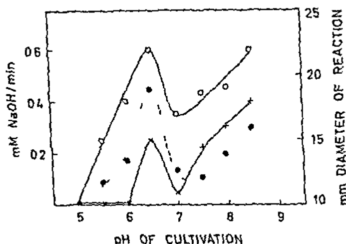


Fig 8
Production of lipase by the Wood strain at various pH ●——● activity on tributyrin in mM NaOH per min per ml mm diameter reaction obtained with egg yolk (○——○) and Tween 60 (×——×)

tent of protein compared with that produced at pH 8 5 (Figs 5 and 6) Thus the specific activity of the preparation was high when produced at pH 5 5

Hyaluronidase The production of hyaluronidase at various pH values was studied and the results are shown in Fig 7 Maximal hyaluronidase values were obtained when the bacteria were grown at pH between 6 0 and 7 0 with a peak activity around 6 5 The presence of glucose in the medium greatly enhanced the production of this enzyme

Lipase The effect of pH of growth on the production of lipase by the Wood strain is illustrated in Fig 8 The presence or absence of glucose

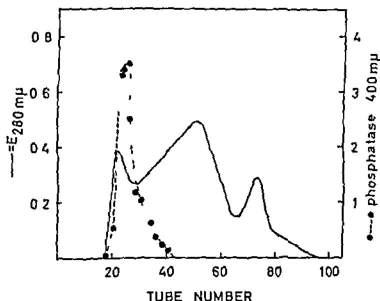


Fig 5

Gelfiltration on Sephadex G 100 of the phosphatase produced by strain Walker at pH 8 5

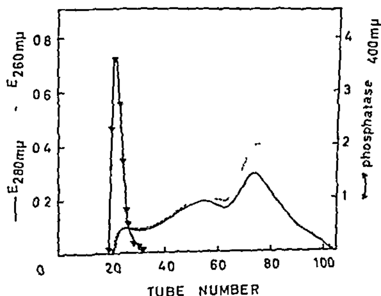


Fig 6

Gelfiltration on Sephadex G 100 of the phosphatase produced by strain Walker at pH 5 5

curred together in a single peak close to the void volume (Figs 5 and 6) Further work is needed to see whether the two activities may be separated by other means It should be noted that the enzyme produced at pH 5 5 and separated on Sephadex G 100 gel had a low con

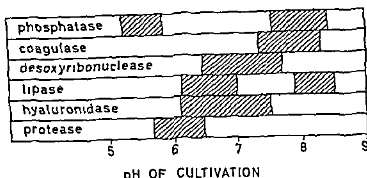


Fig 11

Relationship between pH of growth and production of extracellular enzymes

another above pH 7 5 However no differences in the pH optima for activity was demonstrable

Desoxyribonuclease Although staphylococcal enzymes are known to be active against DNA and RNA the term DNase was used in the present study to denote that the experiments were performed using DNA as the substrate Production of DNase occurred when the bacteria were cultivated at pH values between 6 0 and 8 0 giving maximal yields at pH values between 6 5 and 7 5 (Fig 9)

Protease No proteolytic enzyme was demonstrable when the Wood strain was grown in the presence of glucose On a casein hydrolysate medium the maximum production of protease was found to occur at pH 6 0 (Fig 10)

Comparison of the Production of Different Enzymes at Various pH Values

The relationship between pH of growth and the production of various enzymes can be summarized as shown in Fig 11 At pH values between 6 0 and 7 0 maximal yields of protease hyaluronidase lipase and DNase were obtained Coagulase and phosphatase were produced at pH values above 7 0 Phosphatase was also produced in the acid and lipase in the alkaline pH region

DISCUSSION

The results presented above indicate that the pH at which staphylococci are cultivated exerts a great influence on the biosynthesis or the liberation of the enzymes The experiments do not cover more than two strains and enzymes could be produced by these strains under varied conditions as for example different composition of media Therefore additional experiments seem to be needed before general conclusions can be drawn as to the optimal pH values for their production It seems obvious however that due consideration must be taken to the

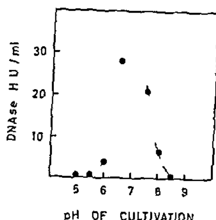


Fig 9

Production of desoxyribonuclease by strain Wood at various pH

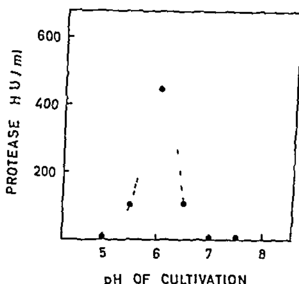


Fig 10

Proteolytic activity in the supernatants of Wood strain grown at various pH values

in the medium did not seem to affect the production of this enzyme as judged by the activity per unit of optical density of the cultures Similar patterns of activity were obtained with the three substrates No activity in the supernatants was demonstrable when the bacteria were grown at pH 5.0 or below The activity gradually increased with the rise in pH of growth reaching a maximal value around pH 6.5 above which the activity decreased Growth at pH values of 7.5 or higher showed again an increase in the production of lipase Thus lipase was produced maximally at two pH regions one at pH 6.5 and

occurrence of such strains was noted in a study comprising 400 clinical strains These results will be published elsewhere

SUMMARY

Two strains of Staphylococcus aureus Wood and Walker were cultivated at constant pH values in the range of 4 5 and 9 0 The effect of such cultivation on the growth and liberation of various extracellular products was studied Between pH 5 0 and 8 5 good growth occurred and addition of glucose resulted in the production of greater number of viable cells Analysis of the supernatants of cultures grown at various pH values indicated that different substances had different pH optima for biosynthesis or liberation into the medium The amount of coagulase in the supernatant decreased when the pH shifted toward the acid side Thus maintenance of pH above 7 is of importance for the performance of routine tests of coagulase activity in the diagnostic laboratory The experimental conditions for the production of other components by the two strains were investigated A great variation in the quantities produced at different pH values was found

REFERENCES

1 *Barnes E H & Morris J F* A quantitative study of the phosphatase activity of *Micrococcus pyogenes* J Bact 73 100 104 1957

2 *Bungay C* The effect of temperature on the production by coagulase positive staphylococci of staphylokinase and other extracellular substances J Gen Microbiol 2, 393–400 1961

3 *Burns J & Holtman D F* Biochemical properties of virulent and avirulent staphylococci Ann N Y Acad Sci *88* 1115–1124 1960

4 *Connon F D & Haun C Y 7* Phosphatase activity of *Staphylococcus aureus* Correlation of enzyme production with resistance to penicillin and phage pattern J Bact 86 105 –1056 1963

5 *Davies G F* Factors influencing the in vitro production of staphylococcal coagulase J Gen Microbiol 5 687 697 1951

6 *Drummond M C & Tager M* Enzymatic activity of staphyl coagulase I Characterization of an esterase associated with purified preparations J Bact 78 407–412 1959

7 *Drummond M C & Tager M* Separation of acid phosphatase from tributyrinase and plasma clotting activities in partially purified staphylocoagulase J Bact 83 432 1962

8 *Flek S D* *Staphylococcus pyogenes* and its relation to disease E & S Livingstone Ltd London 219–343 1955

9 *Gillespie W A & Alder V G* Production of opacity in egg yolk media by coagulase positive staphylococci J Pathol Bact *64* 187–200 1952

10 *Inniss W E & San Clemente C L* Biochemical studies on staphylocoagulase and an allied phosphatase activity J Bact 83 941–947 1962

11 *Kapral F A & Meyer C J* Studies on the induction of *Staphylococcus aureus* alpha toxin Bact Proceed. 66th Ann Meeting *p 44 1966*

12 *Lundbeck H & Tirunarayanan M O* Investigations on the enzymes and toxin of staphylococci Study of the egg yolk reaction using an agar plate assay method Acta path microbiol scandinav *68 123 134 1966*

13 *Lundbeck H & Ask F* Påvisning av stafylokocker i livsmedel Svenska Institutet för konserveringsforskning Rep no 125 16–26 196

14 *Lundbeck H & Ask E* Demonstration of staphylococci in food stuffs (to be published)

15 *Malteaux F J* & *San Clemente C L* Staphylococcal acid phosphatase production and its elution from the cell surface Bact Proceed p 4 19 7
16 *Robinson J* *Thatcher F S* & *Montford J* Studies with staphylococcus toxins V Possible identification of alpha hemolysin with a proteolytic enzyme Can J Microbiol 6 183–194 1960
17 *Soll T* Interrelationship of extracellular enzymes and pseudocapsulation in a strain of *Staphylococcus aureus* J Bact 83 1238–1243 1962
18 *San Clemente C L* & *Zolli Jr Z* Separation of phosphatase from staphylo coagulase preparations J Bact 86 606–608 1963
19 *Tager M* & *Drummond M C* Staphylocoagulase Ann N Y Acad Sci 128 92–111 1965
20 *Tirunarayanan M O* Investigations on the enzymes and toxins of staphylococci Stimulation of coagulase biosynthesis by glucose and bicarbonate Acta path microbiol scandinav 68 273–280 1966
21 *Tirunarayanan M O* & *Lundbeck H* Investigations on the enzymes and toxins of staphylococci Assay of lipase using Tween as the substrate Acta path microbiol scandinav 72 263–276 1968
22 *Tirunarayanan M O* & *Lundbeck H* Investigations on the enzymes and toxins of staphylococci Hydrolysis of triglycerides and other esters by lipase Acta path microbiol scandinav 73 437–449 1968
23 *Tirunarayanan M O* *Lundbeck H* & *Lundblad G* Investigations on the enzymes and toxins of staphylococci Studies on nuclease Acta path microbiol scandinav (To be published)
24 *Tirunarayanan M O* & *Lundblad G* Investigations on the enzymes and toxins of staphylococci Proteolytic enzymes and their purification Acta path microbiol scandinav 68 135 141 1966
25 *Tirunarayanan M O* & *Lundblad G* Investigations on the enzymes and toxins of staphylococci Study of hyaluronidase by the viscosimetric method Acta path microbiol scandinav 73 211–219 1968

Acta path et microbiol scandinav 74 297-300 1968

The National Bacteriological Laboratory Stockholm and the Department of Infectious Diseases Danderyd Hospital Stockholm Sweden

MYCOPLASMA PNEUMONIAE INFECTION IN HOSPITALIZED PATIENTS WITH ACUTE RESPIRATORY ILLNESS

By

GUNNEL BIBERFELD JOHAN STENBECK and TORSTEN JOHNSSON

Received 18 iv 68

The importance of *Mycoplasma pneumoniae* as an aetiologic agent in lower respiratory tract illness has been well documented through epidemiological studies in the United States and in several European countries (*Chanock et al* 1963 *Marmion & Hers* 1963 *Goodburn et al* 1963 *Jansson et al* 1964 *Biberfeld et al* 1965 *Grayston et al* 1965 *Chanock* 1965 *Sobeslavsky et al* 1965 *Alexander et al* 1966 *Krech & Modde* 1966 *Lind* 1966 *Sterner et al* 1966 *Wittleb et al* 1966 *Eng* 1967 *Feizi et al* 1967 *Hornsleth* 1967)

Observations over a period of several years have shown considerable fluctuations in the incidence of *M pneumoniae* infections in some areas in the United States (*Chanock* 1965) However in the Seattle area there was little fluctuation during a three year period (*Alexander et al* 1966) The results of previous investigations indicated that *M pneumoniae* was a common cause of pneumonia in Sweden during the years 1962 and 1963 (*Biberfeld et al* 1965 *Sterner et al* 1966) A more systematic study including isolations and serological tests was initiated in 1964 to follow the incidence of *M pneumoniae* infections over a longer period of time The following report describes the epidemiological serological and clinical results of a two year study of *M pneumoniae* infections among hospitalized patients with acute respiratory illness

MATERIALS AND METHODS

The material studied consisted of 7 2 patients with acute respiratory infections hospitalized during the period February 1964–March 1966 at the department of infectious diseases Danderyd hospital which receives patients from the surroundings of Stockholm Specimens were collected from patients of all ages except children below 5 years of age (Table 8) 377 of the patients studied had a roentgenologically

We wish to thank Mrs *Anna Hammarlund* and Mrs *Inga Haag* who collected the specimens and Mrs *Helen Linder* and Mrs *Kari Ann Sundquist* for skilful laboratory assistance

This work was supported by the *Swedish Medical Research Council* (Project No W 448)

verified diagnosis of pneumonia 52 cases of *cough and fever* without roentgenological changes of the lungs were classified as bronchitis and the remaining 343 cases as upper respiratory tract illness (URI) The laboratory examinations performed *for the diagnosis of M pneumoniae* infection are listed in Table 1 Both isolation assays and complement fixation (CF) tests were done in 511 cases CF tests only were performed in 249 cases and isolation attempts *only in 12 cases* Cold agglutinin reaction was performed with sera from 348 cases of pneumonia *and from* 50 cases of bronchitis Sera from patients with serological evidence of *M pneumoniae infection were also tested for CF antibodies to the following* viral antigens adenovirus influenza A and B parainfluenza 1–3 respiratory syncytial virus psittacosis and mumps virus For various reasons we did not obtain specimens from all cases of respiratory infection in patients above 5 years of age treated at the hospital during the study period In approximately 80 cases of pneumonia (not included among the 377 cases of pneumonia mentioned above) either no specimens or only an acute phase blood specimen was taken

TABLE 1

Laboratory Examinations Performed in 772 Cases of Respiratory Infection for the Diagnosis of M pneumoniae *Infection*

Diagnosis	Isolation assay and CF test No of cases	CF test only No of cases	Isolation assay only No of cases	Total
Pneumonia	255	110	12	377
Bronchitis	28	24	0	52
URI	228	115	0	343
Total	511	249	12	772

URI = upper respiratory illness

In three families where two or more family members *were hospitalized with* respiratory illness caused by *M pneumoniae* the spread of infection was studied

Diagnostic criteria Recovery of the micro organism or demonstration of a four fold or greater increase of CF antibody was taken as diagnostic evidence of *M pneumoniae* infection Patients who had CF titres of $\geq$ 1/64 without a fourfold rise together with a positive cold agglutinin test (fourfold change in titre or titre $\geq$ 1/64) were also classified as cases of recent *M pneumoniae infection*

Collection of specimens As a rule throat washing and swab specimens and a blood sample were taken on the first day after admission of the patient to the hospital The second blood sample was drawn usually 7 15 days later when the patients were *discharged from the hospital Blood specimens were allowed to clot* at room temperature and were never chilled before separation of serum to prevent loss of cold agglutinins

From children 5 9 years old the blood was usually drawn from the finger tip (*Norrby et al* 1963) 0 2 *ml of blood was collected and immediately mixed with* 0 6 ml of phosphate buffered saline (PBS) containing 1 25000 heparin The erythrocytes were removed by low speed centrifugation The supernatant was considered to represent a serum dilution of 1/3

Both a throat washing and a throat swab specimen were collected from most cases Patients were asked to gargle with 5 *ml of saline containing 0 5 per cent* bovine albumin Throat swabs were placed in 2 ml of the same fluid Throat specimens were transported in thermos bottles packed with ice and were either used for isolation the same day or were stored at −65 C

Specimens from the nose and throat were also taken for routine bacteriological examination

Culture medium Agar plates were prepared with 7 parts of Difco PPLO agar 2 parts of unheated horse serum and 1 part of 2 per cent yeast extract as described

by *Chanock et al.* (1962). The medium also contained penicillin (1000 units/ml), amphotericin (5 µg/ml) and thallium acetate (500 µg/ml). Broth medium was prepared in the same way except that Difco PPLO agar was replaced by PPLO broth. Broth used for the preparation of *M. pneumoniae* CF antigen also contained 1 per cent glucose and 0.002 per cent phenol red.

Reference strain. The FH strain of *M. pneumoniae* was obtained from Dr. *Chanock*, NIH, Bethesda.

Isolation of M. pneumoniae. Agar plates and broth were used for isolation. 0.1 ml of throat specimens was inoculated onto each of two agar plates and 0.2 ml of the same material into 2 ml of broth. The inoculated broth and agar plates were incubated at 37°. Subcultures from broth to agar plates were made after incubation for up to three weeks.

Colonies of *M. pneumoniae* were identified by their typical morphology, by the demonstration of haemolytic activity (*Somerson et al.* 1963) and by the fluorescent antibody technique (Coons 1950) as described in detail earlier (*Biberfeld et al.* 1965).

Complement fixation (CF) test. The CF antigen was prepared from the FH strain grown in broth by the method described by *Kenny & Grayston* 1965. The inoculated broth was incubated at 37° on a shaker until the phenol red indicator turned yellow. The culture was centrifuged at 19000 rpm (rotor R 19) in a Spinco ultracentrifuge for 45 minutes, the pellet was washed twice with phosphate buffered saline (PBS) and finally resuspended in PBS at a 100 fold concentration. This concentrated suspension of *M. pneumoniae* organisms was purified by chloroform-methanol extraction. 5 ml of the concentrate were mixed with 100 ml of chloroform and 50 ml of methanol and the mixture was shaken vigorously. 37.5 ml of 0.1 M aqueous KCl were added and the mixture was shaken again. The mixture was kept at +4° until two clear phases formed. The chloroform phase was collected, evaporated and the residue reconstituted with 5 ml of alcohol. 15 ml of 5 per cent bovine albumin in PBS was added to the 5 ml of antigen dissolved in alcohol. The titre of the antigen was determined through a checker board titration.

The CF test was performed in plastic cup trays by the drop method of *Fulton & Dumbell* (1949). Sera were titrated in twofold dilutions, pipettes being changed at each step. 4 units of antigen, 2 units of complement and sheep erythrocytes in a concentration of 0.5 per cent were used. The mixtures of serum dilution, antigen and complement were allowed to bind at +4° overnight before the addition of the haemolytic system.

Cold agglutinin (CA) test. The procedure followed was that described by *Feller & Hilleman* (1956). 0.4 ml of twofold serum dilutions and 0.4 ml of 0.2 per cent suspension of human O erythrocytes were mixed and incubated at +4° C overnight. Titres were expressed as the initial serum dilution of the last tube showing agglutination visible to the unaided eye. A fourfold increase in titre or an unchanging titre of ≥ 1/64 was regarded as positive.

RESULTS

Isolation. *M. pneumoniae* was isolated from 50 patients (19 *per cent*) among 267 cases of pneumonia, from one out of 28 cases of bronchitis and from one out of 228 cases of URI. Thus the total number of isolations was 52. In 47 cases *M. pneumoniae* was recovered upon direct agar plating and in the other 5 cases culture in broth was required. 47 of the isolated strains were identified as *M. pneumoniae* by the indirect fluorescent antibody technique. All these strains produced beta-haemolysis of guinea pig erythrocytes. The other 5 isolations were identified only by typical colony morphology and by production of beta-haemolytic plaques.

From 29 of the patients who yielded *M. pneumoniae* both a throat washing and a throat swab specimen had been collected on the same occasion. In 18 of these cases *M. pneumoniae* was isolated from both

throat specimens in 8 cases from the throat washing only and in 3 cases from the throat swab sample only

In Table 2 is shown the time interval in days between onset of illness and collection of throat samples in cases from which *M pneumoniae* was isolated and in cases that were isolation negative but CI positive

TABLE 2

The Relation between M pneumoniae Isolations and the Time when Throat Specimens were Collected

No of days after onset of illness when throat specimens were collected	No of cases	
	Isolation positive	Isolation negative and CF positive
3– 7	24	4
8–14	22	5
15–21	6	3
≧ 22		2

TABLE 3

Distribution of CF Antibody Titres in the First and Second Serum Samples Taken from 85 Cases of Pneumonia Serologically Diagnosed as M pneumoniae Infection The Titres are Expressed as the Reciprocal of the Dilution

First sera CF titre	Second sera CF titre									
	<4	4	8	16	32	64	128	256	512	1024
<4			4	4	4	4	8	2		1
4						2	1			–
8					1	1	5		2	
16						2	3	3	2	
32							3	3	1	1
64						2	2	1	2	
128						1	2	1	1	–
256							3	6	2	1
512								3	1	

Serological results A fourfold or greater increase in CI antibody to *M pneumoniae* was demonstrated in 62 out of 365 (17 per cent) patients with pneumonia 23 cases had a CI titre of ≧ 1/64 without a fourfold rise All of these 23 cases had also a positive CA reaction Thus 85 (23 per cent) among 365 cases of pneumonia showed serological results indicating a recent *M pneumoniae* infection The distribution of CF antibody titres in these cases is shown in Table 3 The geometric mean titre of the convalescent phase sera was 1/128

Only two out of 52 patients with bronchitis had a fourfold increase in CF antibody A third case showed a twofold increase from 1/64 17 days after onset to 1/128 42 days later followed by a fourfold decrease in titre three months later

In none of the 343 cases of URI was a fourfold rise of CF antibody demonstrated A titre of 1/128 was found in one patient with stomatitis pharyngitis and exanthema Three cases of mononucleosis with a positive Paul Bunnell test also had high CF and cold agglutinin titres (≧ 1/64) without rise (see discussion)

The distribution of CF antibody titres according to age in the convalescent phase sera in 631 cases of respiratory infection without laboratory evidence of recent *M pneumoniae* infection as defined above is shown in Table 4 191 (30 per cent) of these cases had titres between 1/4–1/32 The possibility exists that some of these cases had had a recent infection with M pneumoniae although the CF titres were low and a fourfold rise was not demonstrated The presence of CF antibody was most frequent in the age groups 15–19 and 20–29 years

TABLE 4

CF Antibody Titres against M pneumoniae in 631 Cases of Respiratory Infection without Laboratory Evidence of Recent M pneumoniae Infection The Titres are Expressed as the Reciprocal of the Dilution

Age years	CF antibody titre					Total	% with antibody
	<4	4	8	16	32		
10–14	24	3	4	–	1	32	25
15–19	57	23	12	8	4	104	45
20–29	57	18	25	13	3	116	51
30–39	54	5	6	3	–	68	21
40–49	51	10	4	2	1	68	29
50–59	43	1	5	4	1	68	37
60–69	62	5	4	–	1	72	14
≧ 70	92	9	1	–	1	103	11
Total	440	88	61	30	12	631	30

Cold agglutinins (CA) 93 out of 348 cases of pneumonia were positive in the CA test in 52 of the cases combined with a *fourfold change in titre* and in 41 with an unchanging titre of ≧ 1/64 75 per cent of these 93 patients had a fourfold rise or a high titre of CF antibodies to *M pneumoniae* 80 per cent of 61 cases with a fourfold increase of CF antibodies and all of the cases with CF titres of ≧ 1/64 without a rise were positive in the CA test The three CF positive cases of bronchitis were all CA negative

Correlation between isolation and serological results The relationship of isolation to CF antibody titres in patients with pneumonia is shown in Table 5 *M pneumoniae* was isolated from 34 out of 44 (77 per cent) patients with a fourfold rise of CF antibodies and from 11 out of 14 (79 per cent) cases with a high CF *titre (≧ 1/64) without rise* The organism was also recovered from 4 of 50 (8 per cent) patients with CF titres 1/4 to 1/32 without a fourfold rise but from none of the

147 cases without demonstrable CF antibodies Isolation was successful in one of the 12 cases where CF antibody test was not performed As regards 18 cases with a fourfold CF antibody rise and 9 cases with a high CF titre (≧ 1/64) without rise throat specimens were not available

TABLE 5

Correlation between the Isolation Results and CF Antibody Titres to M pneumoniae *in Patients with Pneumonia*

	Isolation of *M pneumoniae* Pos No	Neg No	Total No	% with pos isolation
Fourfold CF antibody rise	34	10	44	77
CF titre ≧ 1/64 no rise	11	3	14	79
CF titre 1/4–1/32 no rise	4	46	50	8
CF titre $< 1/4$	0	147	147	0
Total	49	206	255	

TABLE 6

Criteria of M pneumoniae *Infection in 90 Cases of Pneumonia*

	No of cases
Recovery of M pneumoniae and fourfold CF antibody rise	34
Fourfold CF antibody rise	28
Recovery of M pneumoniae and CF titre ≧ 1/64 without rise	11
CF titre ≧ 1/64 without rise and CA positive	12
Recovery of M pneumoniae CF titre low without rise	4
Recovery of M pneumoniae (CF and CA not done)	1
Total	90

(CF = complement fixation CA = cold agglutination)

The number of pneumonia cases diagnosed as *M pneumoniae* infection and the laboratory results upon which the diagnosis was based are presented in Table 6 Altogether 90 patients with pneumonia were classified as cases of recent *M pneumoniae* infection

Three cases of bronchitis had CF antibody titres compatible with recent *M pneumoniae* infection Isolation was successful in one of the two cases from which material was available

There was only one case of URI with evidence of *M pneumoniae* infection This case yielded *M pneumoniae* and had a CF antibody titre of 1/128 without rise

Clinical findings The symptoms in cases of pneumonia associated with *M pneumoniae* infection are shown in Table 7 The onset of illness was mostly acute with chills *high fever and cough but in 17 per*

cent of the cases symptoms developed slowly over 2 days or more. Fever above 39.0 and cough were present in all cases. Headache and chills were frequent. The average duration of fever above 37.5 C was 10 days. The physical findings in the lungs were often discrete in the beginning of illness but rales developed in 82 per cent of the cases during the course of illness. All cases of pneumonia were diagnosed by x ray. The roentgenological changes in the lungs were unilateral in 52 cases (58 per cent) and bilateral in 38 cases. The x ray pictures were variable and showed no characteristic diagnostic features. The erythrocyte sedimentation rate (ESR) was elevated in all but 2 cases. Values over 50 mm per hour were found in two thirds of the patients. The white blood cell count was generally normal; in only 11 cases (12 per cent) leucocytosis (> 10000 cells/mm³) was found and leucopenia (< 3000 cells/mm³) was not seen in any case.

TABLE 7
Symptoms and Clinical Signs in 90 Cases of Pneumonia Associated with M pneumoniae *Infection*

	No of cases	%
Cough	90	100
Headache	65	72
Chills	66	73
Nausea vomiting	40	44
Sore throat	37	41
Nasal symptoms	15	17
Fever ≧ 39.0 C	90	100
Rales	74	82
Pharyngeal erythema	51	57
Cervical adenopathy	25	28

Before admission to the hospital most pneumonia patients had been treated with antibiotics: 70 per cent had received penicillin or sulpha and 13 per cent had received tetracyclines. At the hospital the patients usually were treated with demethylchlortetracycline (Ledermycin). In 13 cases penicillin only was used. The average duration of hospital treatment was 11 days.

Stomatitis without pneumonia was found in an 11 year old girl. She had fever for four days, a sore throat, vesiculae in the mouth and a maculo papular exanthema on the body. The CF antibody titre was 1/128 eight days after the onset, being 1/64 one month later. *M pneumoniae* was isolated from the throat. CF antibody titres against herpes simplex, adenovirus, respiratory syncytial virus, influenza A and B and parainfluenza 1–3 were negative. No pathogenic bacteria were isolated.

Complications. In 7 cases of pneumonia exanthema was noticed, which in 5 of the cases was mild and disappeared within a few days

The other two patients who had erythema nodosum like exanthema had a prolonged illness with fever for 17 and 20 days and a high ESR at 121 and 130 mm respectively 4 cases with otitis were recorded 2 cases had gastroenteritis In one case a 23 year old man meningo encephalitis with slow cerebration bladder and gutparesis and pathological electroencephalogram developed 10 days after the onset of pneumonia The cerebrospinal fluid (CSF) contained 70 leucocytes per mm³ with 34 per cent mononuclear cells and 75 mg per cent protein The CF antibody titre to *M pneumoniae* was 1/128 and the cold agglutinin titre 1/512 in sera taken 10 and 16 days after onset of illness Throat specimens were not obtained Virus isolation from CSF and faeces was negative Isolation of *M pneumoniae* from CSF was not attempted The patient recovered completely

Double infections 2 cases of pneumonia with a significant rise in CF antibodies to *M pneumoniae* had concomitant staphylococcus septicaemia They both recovered In the other cases of *M pneumoniae* infection the bacterial findings from the nose and throat did not appear to have a pathological significance There were three cases with serological evidence of recent infection with adenovirus In addition one case with a high titre against influenza B (≧ 1/256) one case with rise of CF titre against parainfluenza 2 and 3 and one case with rise against mumps virus were encountered Finally one patient had varicella that was followed by *M pneumoniae* pneumonia and otitis

Age and sex distribution The age distribution of the patients with pneumbonia is shown in Table 8 Pneumonia associated with *M pneumoniae* was most common (48 per cent) in the age groups 10–14 and 15–19 years In adults 20 49 years old the incidence was also high (33 per cent) whereas in patients more than 60 years old infection with *M pneumoniae* was rare

TABLE 8

Age Distribution of Patients with Pneumonia Associated with M pneumoniae *Infection*

Age in years	No of cases tested	No of positive cases	%
0– 4	0	0	
5– 9	15	2	13
10–14	18	9	50
15–19	42	20	48
20 29	54 } 156	17 } 51	31 } 33
30–39	53	21	40
40–49	49	13	27
50–59	50	6	12
60–69	40	2	5
≧ 70	56	0	0
Total	377	90	24

188 of the pneumonia cases included in the study were females and 189 were males There were more positive cases among females (53 cases) than among males (37 cases)

The seasonal distribution is shown in Table 9 *M pneumoniae* infections occurred during all seasons

TABLE 9

Seasonal Incidence of Pneumonia Associated with M pneumoniae *Infection*

	1964			1965			1966		
	No of cases tested	No of positive cases	%	No of cases tested	No of positive cases	%	No of cases tested	No of positive cases	%
Jan March	33	8	24	54	9	17	46	10	22
April-June	47	13	28	38	4	11			
July Sept	32	8	25	26	9	35			
Oct Dec	58	18	31	43	11	26			
Total	170	47	28	161	33	20	46	10	22

January 1964 was not included in the study

Family infections Spread of infection within the family was observed in three instances In family L all three members mother and two daughters 10 and 12 years old had pneumonia and were admitted to the hospital The 12 year old daughter fell ill first the mother and the second daughter 19 days later The daughters showed a fourfold increase of CF antibody whereas the mother had a CF titre of 1/16 in paired sera taken 3 and 24 days after onset of illness The latter however had a 64 fold rise in antibodies to *M pneumoniae* measured with the tetrazolium reduction inhibition (TRI) test (*Jensen* 1964 *Taylor Robinson et al* 1966) In family N 4 among 5 members had acute respiratory infection The first case was a 17 year old son who had pneumonia treated at home A few weeks later the mother and the 13 year old son fell ill on the same day The mother developed pneumonia and was admitted to the hospital whereas the son who had bronchitis was treated at home 8 days later the 22 year old daughter fell ill with pneumonia and was also sent to the hospital The mother had CF titres 1/256 12 days after clinical onset and 1/128 7 days later the daughter presented a fourfold rise of CF antibodies In a third family of 6 members two of these the mother and one son developed pneumonia within 5 days and were hospitalized The rest of the family had no overt illness The mother had a significant rise of CF antibodies and the son presented CF titres of 1/80 and 1/160 3 and 11 days after onset of illness *Mycoplasma pneumoniae* was isolated from both

The other two patients who had erythema nodosum like exanthema had a prolonged illness with fever for 17 and 20 days and a high ESR at 121 and 130 mm respectively. 4 cases with otitis were recorded. 2 cases had gastroenteritis. In one case a 23-year-old man meningo-encephalitis with slow cerebration, bladder and gutparesis and pathological electroencephalogram developed 10 days after the onset of pneumonia. The cerebrospinal fluid (CSF) contained 70 leucocytes per mm^3 with 34 per cent mononuclear cells and 75 mg per cent protein. The CF antibody titre to *M pneumoniae* was 1/128 and the cold agglutinin titre 1/512 in sera taken 10 and 16 days after onset of illness. Throat specimens were not obtained. Virus isolation from CSF and faeces was negative. *Isolation of M pneumoniae from CSF* was not attempted. The patient recovered completely.

Double infections. 2 cases of pneumonia with a significant rise in CF antibodies to *M pneumoniae* had concomitant staphylococcus septicaemia. They both recovered. In the other cases of *M pneumoniae* infection the bacterial findings from the nose and throat did not appear to have a pathological significance. There were three cases with serological evidence of recent infection with adenovirus. *In addition one* case with a high titre against influenza B (≥ 1/256), one case with rise of CF titre against parainfluenza 2 and 3 and one case with rise *against mumps virus were encountered. Finally one patient had varicella* that was followed by *M pneumoniae* pneumonia and otitis.

Age and sex distribution. The age distribution of the patients with pneumonia is shown in Table 8. *Pneumonia associated with M pneumoniae* was most common (48 per cent) in the age groups 10–14 and 15–19 years. In adults 20–49 years old the incidence was also high (33 per cent) whereas in patients more than 60 *years old infection* with *M pneumoniae* was rare.

TABLE 8
Age Distribution of Patients with Pneumonia Associated with M pneumoniae *Infection*

Age in years	No of cases tested		No of positive cases		%	
0– 4	0		0			
5– 9	15		2		13	
10–14	18		9		50	
15–19	42		20		48	
20–29	54	156	17	51	31	33
30–39	53		21		40	
40–49	49		13		27	
50–59	50		6		12	
60–69	40		2		5	
≥ 70	56		0		0	
Total	377		90		24	

with cold agglutinins with a titre of 1/64 or higher were also classified as cases of *M pneumoniae* infection Since a CF titre of 1/64 can be found as late as 12 months after onset of illness (*Biberfeld* 1968) we required the concomitant presence of cold agglutinins which decrease fairly rapidly after infection (*Finland et al* 1945) to make a diagnosis of recent *M pneumoniae* infection However in three Paul Bunnell positive cases of infectious mononucleosis the presence of cold agglutinins together with high (1/64) unchanging titres of CF antibody to *M pneumoniae* was not considered sufficient evidence of recent *M pneumoniae* infection since cold agglutinins often appear also in mononucleosis (*Kostinas & Cantow* 1966 *Wager* 1968)

The incidence of cold agglutinins in pneumonia due to *M pneumoniae* has varied in different studies probably due to technical differences or to differences as to time after onset of illness when the specimens were collected (*Chanock et al* 1961 *Jansson & Wager* 1964 *Grayston et al* 1965 *Griffin & Crawford* 1965) In a previous study during which many of the sera had been sent as routine specimens cold agglutinins were demonstrated in 46 per cent of cases of pneumonia caused by *M pneumoniae* (*Biberfeld et al* 1965) In the present study in which special care was taken to avoid chilling of blood samples before the separation of sera and the cold agglutination test was made more sensitive by the use of a 0 2 per cent instead of a 1 per cent suspension of erythrocytes (*Feller & Hilleman* 1956) cold agglutinins were found in 80 per cent of pneumonia cases with a fourfold rise of CF antibodies to *M pneumoniae* However 25 per cent of the total number of pneumonia cases with a positive cold agglutinin test were negative in the CF test

In our opinion the demonstration of cold agglutinins although not pathognomonic for *M pneumoniae* infection is a useful complement to the CF test particularly for serologic diagnosis in cases where the blood samples have been collected too late after onset to allow the demonstration of a fourfold rise of CF antibodies Several of the patients comprised in the present study had been admitted to the hospital rather late in their illness and 30 per cent of the pneumonia cases serologically diagnosed as *M pneumoniae* infection had CF antibody titres and cold agglutinin titres of ≧ 1/64 in the first blood specimen taken the day after admission Thus in these cases a diagnosis of probable *M pneumoniae* infection could be made already one to two days after admission

The proportion of isolations to positive serological reaction (77 per cent) was the same in our investigation as in that by *Grayston et al* (1965) The same kind of *M pneumoniae* CF antigen was used in both studies 90 per cent of the 52 isolations made by us were recovered by direct agar plating and the rest after culture in broth *Grayston et al* obtained 45 per cent of their isolations directly on agar and in the other cases cultivation in diphasic media (broth over agar) was required

The clinical findings in the cases of *M pneumoniae* discussed in this study are in general agreement with those described in previous studies (*Mufson et al* 1961 *Jansson et al* 1961 *Grayston et al* 1965 *Biberfeld et al* 1965) Otitis and meningoencephalitis occurred as complications to pneumonia in a few cases These clinical manifestations have been noted previously in association with *M pneumoniae* infection (*Rifkind et al* 1962 *Sobeslavsky et al* 1965b *Biberfeld et al* 1965 *Skoldenberg* 1965 *Taylor et al* 1967)

SUMMARY

Sera from 760 cases of acute respiratory illness hospitalized during the period February 1964–March 1966 were examined by complement fixation test against *Mycoplasma pneumoniae* antigen Isolation of *M pneumoniae* was attempted in 523 cases 23 per cent of 365 cases of pneumonia and 6 per cent of 52 cases of bronchitis were diagnosed serologically as *M pneumoniae infections* *Only one among 343 cases of* upper respiratory illness had laboratory evidence of infection with *M pneumoniae* *M pneumoniae* was isolated from 19 per cent of 267 patients with pneumonia Isolation was successful in 77 per cent of CF positive cases Altogether 24 per cent of 377 cases of pneumonia were diagnosed serologically and/or by isolation as *M pneumoniae* infections The incidence was highest in the age group 10–19 years where half of pneumonia cases were associated with *M pneumoniae* infection In adults 20–49 years old the incidence was 33 per cent

REFERENCES

Alexander F R Foy H M Kenny C F Kronmal R A McMahon R Clarke E R MacColl W A & Grayston J T Pneumonia due to *Mycoplasma pneumoniae* New Engl J Med *275* 131–136 1966

Biberfeld G Johnsson T & Jonsson J Studies on *Mycoplasma pneumoniae* infection in Sweden Acta path microbiol scandinav *63* 469–475 1965

Biberfeld G Distribution of antibodies within 19S and 7S immunoglobulins following infection with *Mycoplasma pneumoniae* J Immunol *100* 338–347 1968

Biberfeld G & Sterner G unpublished data

Chanock R M Mufson M A Bloom H H James W D Fox H H & Kingston J R Eaton agent pneumonia J A M A *175* 213 220 1961

Chanock R M Hayflick L & Barile M F Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO Proc Nat Acad Sci *48* 41–49 1962

Chanock R M Mufson M A Somerson N L & Couch R B Role of Mycoplasma (PPLO) in human respiratory disease Amer Rev Resp Dis Suppl *88* 218–231 1963

Chanock R M Mycoplasma infections in man New Engl J Med *273* 1257–1264 1965

Coons A H & Kaplan M H Localization of antigen in tissue cells II Improvements in a method for the detection of antigen by means of fluorescent antibody *J* Exp Med *91* 1–13 1950

Eng J Studies on the complement fixation test with *Mycoplasma pneumoniae* antigen 2 Application of the test to hospitalized pneumonia patients and to healthy blood donors Acta path microbiol scandinav *71* 265–273 1967

Feller A E & Hilleman M R Diagnostic procedures for virus and rickettsial diseases American public health association New York 1956 p 272
Fei i T Maclean H Sommerville R G & Selwyn J G Studies on an epidemic of respiratory disease caused by *Mycoplasma pneumoniae* Brit Med J *1* 457–460 1967
Finland M Peterson O L Allen H E Samper B A & Barnes M W Cold agglutinins II Cold isohemagglutinins in primary atypical pneumonia of unknown etiology with a note on the occurrence of hemolytic anemia in these cases J Clin Invest *24* 454–473 1945
Fulton F & Dumbell K R The serological comparison of strains of influenza virus J Gen Microbiol *3* 97–111 1949
Goodburn G M Marmion B P & Kendall E J C Infection with Eaton s primary atypical pneumonia agent in England Brit Med J *1* 1266–1270 1963
Grayston J T Alexander E R Kenny G E Clarke E R Fremont J C & MacColl W A *Mycoplasma pneumoniae* infections Clinical and Epidemiologic studies J A M A *191* 369–374 1965
Griffin J P & Crawford Y E *Mycoplasma pneumoniae* in primary atypical pneumonia J A M A *193* 1011–1016 1965
Hornsleth A *Mycoplasma pneumoniae* infection in infants and children in Copenhagen 1963–1965 Acta path microbiol scandinav *69* 304–313 1967
Jansson E Wager O Stenström R Klemola E & Forsell P Studies on Eaton PPLO pneumonia Brit Med J *1* 142–145 1964
Jensen K E Antibodies to *Mycoplasma pneumoniae* measured by inhibition of tetrazolium reduction Bact Proc p 70 1964
Kenny G E & Grayston J T Eaton pleuropneumonia like organism (Mycoplasma pneumoniae) complement fixing antigen Extraction with organic solvents J Immunol *95* 19–25 1965
Kostinas J E & Cantow E F Studies on infectious mononucleosis I Antibodies Amer J Med Sci *252* 721–725 1966
Krech U & Modde H Untersuchung über Haufigkeit und Bedeutung von Infektionen mit *Mycoplasma pneumoniae* Deutsche Med Wschr *91* 1013–1015 1966
Lind K Isolation of *Mycoplasma pneumoniae* (Eaton agent) from patients with primary atypical pneumonia. Acta path microbiol scandinav *66* 124–134 1966
Marmion B P & Hers J F Ph The Mycoplasma (PPLO) agents Observations on Eaton primary atypical pneumonia agent and analogous problems in animals Amer Rev Resp Dis Suppl *88* 198 211 1963
Mufson M A Manko M A Kingston J R & Chanock R M Eaton agent pneumonia clinical features J A M A *178* 369–374 1961
Norrby E Lagercrant R Gard S & Carlström G Measles vaccination I Serologic response to an inactivated vaccine Arch Ges Virusforsch *13* 538 558 1963
Rifkind D Chanock R M Kravet H M Johnson K M & Knight V Ear involvement (myringitis) and primary atypical pneumonia following inoculation of volunteers with Eaton agent Amer Rev Resp Dis *85* 479 1962
Sköldenberg B Aseptic meningitis and meningoencephalitis in cold agglutinin positive infections Brit Med J *1* 100–102 1965
Sobeslavsky O Syrucek L Bruchova M Herdegen L Stichenwirthova B Zapletal A Samankova L Danesova J Abrahamovic M Krejci D & Prkova Z A contribution on the ecology of *Mycoplasma pneumoniae* infections J H g Epidem 9 86–94 1965a
Sobeslavsky O Syrucek L Bruckova M & Abrahamovic M The etiological role of *Mycoplasma pneumoniae* in otitis media in children Pediatrics *3* 652–657 1965b
Somerson N L Taylor Robinson D & Chanock R M Hemolysin production as an aid in the identification and quantitation of Eaton agent (*Mycoplasma pneumoniae*) Am J Hyg 77 122–128 1963
Sterner G Swedmyr A Tunevall G & Volontis S Infections with Eaton agent in pneumonia Acta Med Scandinav *178* 751–757 1966
Taylor M J Burrow G N Strauch B & Horstman D M Meningoencephalitis associated with pneumonitis due to *Mycoplasma pneumonia* J A M A *199* 8 3–816 1967

Taylor Robinson D Sobeslavsky O Jensen K E Senterfit L B & Chanock R M Serologic response to *Mycoplasma pneumoniae* infection I Evaluation of immunofluorescence complement fixation indirect hemagglutination and tetrazolium reduction inhibition tests for the diagnosis of infection Amer J Epidemiol *83* 287-298 1966
Wager O Personal communication 1968
Weileb B *Sprössig M* *Anger G* & *Heidler S* Respiratory disease due to Mycoplasma pneumoniae German Medical Monthly *XI* 345-348 1966

Acta path et microbiol scandinav 74 301-302 1968

BRIEF REPORT

THE IN VITRO UPTAKE OF OESTRADIOL IN BIOPSIES FROM 25 BREAST CANCER PATIENTS

By Sten Sander

Hormone responsive and hormone unresponsive breast cancers cannot be distinguished by histological examination and currently there is no practical way of predicting whether one or another patient will respond to hormonal treatment. There is however some evidence that breast cancers which respond favourably to hormonal treatment possess a relatively high affinity to oestrogens (?). We also know from experiments with chemically induced breast tumours in rats that there is a correlation between the uptake of oestradiol 17β and the response to oophorectomy (4 5).

If the uptake of oestradiol in breast cancer tissue yields information about endocrine responses then uptake studies are a possible approach to a selection of patients who will respond favourably to endocrine treatment.

This presentation is concerned with the *in vitro* uptake of oestradiol in human breast cancers measured in slices of incubated tumour tissue.

The investigation was carried out at the Norwegian Radium Hospital during August/September 1967. None of the 25 patients had received previous treatment for breast cancer. Radical mastectomy was performed in all cases and specimens from the primary tumour and muscle controls were taken out for further examination. Immediately after excision of the breast tumour pieces of tumour and muscle tissue were put separately in vials containing Krebs Ringer solution. The tissues were brough without delay to the laboratory and sliced on a Stadie Riggs handmicrotome. Great care was taken to excise necrotic or scarred parts of the tumour before slicing. The tumour slices were incubated in freshly prepared Krebs Ringer phosphate buffer containing 4×10^{-5} µg oestradiol (6 7-H³) per ml of medium. Three parallels (60 70 mg of tissue) from each tumour were run. The incubation period was ? hours at 37 C. Skeletal muscle was incubated to serve as control. The incubation was terminated by removal of the slices from the medium the slices were briefly rinsed in Tris acetate buffer pH 7 4 and blotted dry. Subsequently the slices were homogenized by ultrasonic disintegration in 0 5 N NaOH. Aliquots were taken from the homogenate for measurement of radioactivity and for protein determination. Radioactivity was estimated in a liquid scintillation system. Naphthalene 80 g PPO 5 g POPOP 0 05 g Formic acid 10 g in ethanol 230 ml dioxan 385 ml and xylol 38 ml. The samples were counted 24 hours after preparation in a Nuclear Chicago Liquid Scintillation Counter mark 1 model 6860. Protein determination was performed on diluted aliquots by the method of *Lowry et al* (3).

Concentration of radioactivity in the tumour tissue and in the muscle controls was expressed as DPM per mg of protein. The uptake capacity of a tumour was expressed as the ratio between the concentration of radioactivity in tumour and muscle control.

Tissue for histological examination was embedded in *paraffin* and sections stained with haematoxylin/eosin.

The capacity of tumours to take up oestradiol is shown in Fig 1. It will be seen that some of the tumours display a high uptake capacity while others do not concentrate the labelled steroid significantly.

It may be said that our *measurements divide* the tumours into two populations. 9 of the examined tumours show a distinctly higher oestradiol uptake than the rest

---

Received 13 viii 68 from the Department of Surgical Research Rikshospitalet Oslo and the University Institute for Pathological Anatomy Rikshospitalet Oslo Norway

The author wishes to express his thanks to Chief surgeon *I O Brennhovd* for his kind support. This work has been supported by a grant from *The Norwegian Research Council for Science and the Humanities*

Taylor Robinson D Sobeslavsky O Jensen K F Senterfit L B & Chanock R M Serologic response to *Mycoplasma pneumoniae* infection I Evaluation of immunofluorescence complement fixation indirect hemagglutination and tetrazolium reduction inhibition tests for the diagnosis of infection Amer J Epidemiol *83* 287 298 1966
Wager O Personal communication 1968
Witleb W *Sprossig M* *Anger C* & *Heidler S* Respiratory disease due to Mycoplasma pneumoniae German Medical Monthly *XI* 345–348 1966

Acta path et microbiol scandinav 74 303-304 1968

BRIEF REPORT

TRANSFER AMYLOIDOSIS ULTRASTRUCTURE OF THE TRANSFERRED SUBCELLULAR FRACTIONS

By *John Wanstrup* & *Poul Ranløv*

The adoptive transfer of experimentally induced amyloidosis between syngeneic mice using cell suspensions from casein treated donor mice (*Werdelin & Ranløv* 1966) or spleen cell homogenates (fractionated) obtained from similarly pretreated donor mice (*Ranløv* 1967) has recently been described The conclusion to be drawn from the latter experiment lends support to the assumption of a messenger function operating in this transfer system and being located to the "DNA protein" fraction (*Medawar* 1963) of the subcellular homogenate from the presensitized spleens

The present report deals with the ultrastructure of the sub cellular fractions of the transfer system reported by *Ranløv* (1967)

Experimental Procedures

20 randomized C3H mice were pre immunized with 17 daily casein injections The spleens (total weight 3 0 g) were treated as previously described in order to obtain a suspension of crushed cells (*i e* treatment in a Potter Elvehjem homogenizer repeated washing in a solution of cold isotonic saline and distilled water repeated homogenization in a Waring blender at top speed ultrasonic irradiation and finally centrifugation at 5 000 g for 10 minutes in the cold) The final amyloidogenic sediment "DNA protein" (*Medawar* 1963) was prefixed in glutaraldehyde 3 per cent after fixed in OsO_4 and embedded in Vestopal W Ultrafine sections were cut on an LKB ultratome stained with lead hydroxide and uranyl acetate and investigated in a Siemens Elmiskop I The non amyloidogenic supernatant was centrifuged at 35 000 g for one hour and the resulting sediment prepared in a similar way for investigation in the electron microscope This latter fraction is termed "Antigenic sediment" according to *Medawar* (1963)

Results and Comments

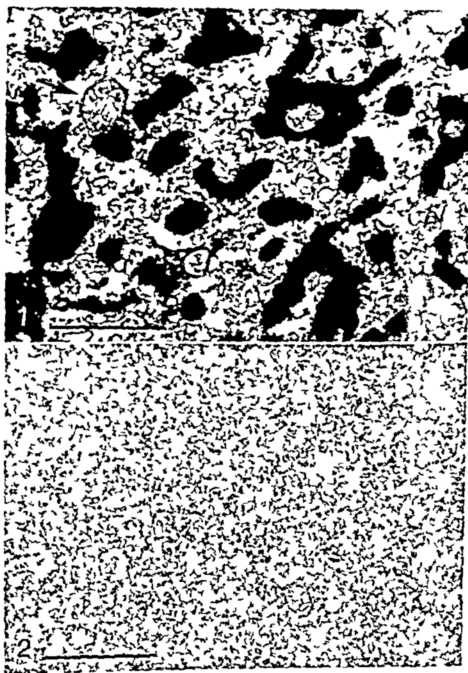
The DNA protein fraction Ultrastructurally this sediment appears as a heterogeneous substance made up of more or less recognizable nuclear and cytoplasmic components (Fig 1) The dominating structure is strongly osmiophilic non membrane lined aggregations of a granular material From its morphology and the fact that this fraction is strongly Feulgen positive it seems justified to interpret these structures as aggregations of nuclear chromatin These conspicious structures are dispersed in a mainly granular "matrix" containing a number of lamellar and vesicular bodies some of which are coated with ribosomes Occasionally a single mitochondrion has survived the experimental procedures (arrow) Sometimes small bundles of amyloid are seen interstitially This is to be expected since 5-10 per cent of the donor animals showed minor degrees of spleen amyloidosis following the casein treatment No intact cells or larger cell fractions appeared in this sediment.

Antigenic sediment fraction This non amyloidogenic fraction appears quite different from the one described above It appears more homogeneous composed of non identifiable granular and vesicular material However evenly dispersed larger and smaller clusters of probably free ribosomes or polysomes are seen No other cytoplasmic or nuclear constituents could be recognized (Fig 2)

The present preliminary study allows the conclusion to be drawn

1) That we are in fact dealing with a subcellular transfer system in the experimental mouse amyloidosis as no intact cells were present in the investigated fractions

Received 17 viii 68 from *the University Institute of Pathological Anatomy (Head Professor G Teilum M D) Copenhagen Denmark*



2) It is tempting to regard the dominating nucleus derived materials in the DNA protein" fraction as the morphologic expression of the amyloidosis inducing agent or the carrier of this agent which

3) inspire to further attempts to isolate the active agent

References *Medawar P B* Transpl *1* 21 1963—*Ranlov P* Acta path micro biol scandinav *70* 321 1967—*Werdelin O & Ranlov P* Acta path microbiol scandinav *68* 1 1966

Acta path microbiol scandinav 74 305–310 1968

The Department of Pathology Karolinska sjukhuset Stockholm Sweden (B R)
The Department of Medicine Lasarettet Sodertalje Sweden (L L)

HUMAN TARTRATE NEPHROPATHY

Report of a Fatal Case

By

BENGT ROBERTSON and LENNART LONNELL

Received 23 xii 66

Tartaric acid and its potassium and sodium salts are frequently used in baking and are common constituents of effervescent powders (11) They also have a medical use as cathartics (oral dose 0 3–2 g) (3 7 11) No toxic effects on the human kidney have been observed after the administration of therapeutic doses (10) Larger doses however are said to cause renal damage (3 7 11) The lethal dose of tartaric acid is estimated as 15–30 g (1 2 6 8) and according to *Underhill et al* (13) fatal instances of tartaric acid poisoning have been reported This statement was not supported by any references and since we have been unable to find any description of human tartrate nephritis in the recent literature the following case report seems to be justified

CASE REPORT

Clinical History

This report concerns a 51 year old confectioner previously in good health except for a gastric ulcer for which he was successfully operated on twelve years prior to his terminal illness Eight days before his final admission he accidentally consumed some gulps of a strong (probably 50 per cent) aqueous solution of tartaric acid which was kept in a lemonade bottle in a refrigerator The amount of tartaric acid ingested was estimated as approximately 30 g Noticing the strong acid taste the confectioner realized his mistake but he ignored the symptoms developing during the next 24 hours i e diarrhoea and bilateral lumbar pain His condition gradually deteriorated but he did not seek medical advice On admission he was oliguric and moribund He died within a few hours after admission just as peritoneal dialysis was being started

An ante mortem urine specimen had a specific gravity of 1 008 contained protein and glucose but had a normal sediment Acidosis and considerable hyperkaliaemia (7 2 mEq/l) an increase in the serum creatinine level to 20 5 mg% and an ESR of 77 mm/h were demonstrated in a blood sample

Autopsy Findings

Autopsy was performed 48 hours post mortem The predominant lesions were found in the kidneys The latter were of equal size and their combined weight was 355 g The outer cortex of both kidneys contained multiple yellow areas visible from the smooth surface as irregularly rounded patches varying in size up to about 3 cm in diameter and bounded by a dark red and apparently haemorrhagic zone



Fig 1

Kidney with cortical infarcts with haemorrhagic borders

Their appearance suggested infarcts the inner border of these areas however ran through the mid-cortex more or less parallel to the kidney surface (Fig 1) The rest of the renal parenchyma was paler than normal with a distinct corticomedullary border The renal pelves were normally wide without evidence of inflammation The urinary bladder was not remarkable No thrombi or any mural lesions were visible in the main renal arteries or veins There was no narrowing of the aortic orifices of the renal arteries

In the gastro intestinal tract there was surgical absence of the distal part of the stomach with gastro enteroanastomosis (Billroth II) The jejunum was moderately distended but there was no macroscopical evidence of enteritis

Each pleural cavity contained 500 cc of clear fluid There was moderate pulmonary oedema The coronary arteries and the aorta showed only slight atherosclerosis otherwise the cardiovascular system was normal with a heart weight of 280 g Other organs were not remarkable

Specimens for histologic examination were taken from the kidneys liver spleen myocardium and pancreas

On routine paraffin sections the following stains were used haematoxylin and eosin van Gieson Ladewig von Kossa the murexide method for calcium and the PAS modification suggested by *Carr* (4) for tartaric acid among other things

Microscopical Findings

The renal lesions were localized mainly to the cortex The yellow zones observed at autopsy corresponded to areas of complete necrosis The centre of these areas was devoid of any cellular reaction whereas the margin were heavily infiltrated by lymphocytes and neutrophil granulocytes (Fig 2) Particularly at the inner margin of the infarcts there was precipitation of a finely granular substance which stained violet in PAS sections (Fig 3 A) The granular material remained unstained in von Kossa and in murexide stained sections At the border of the infarcts there were intramural oedema and necrosis in the walls of many interlobular arteries and arterioles and many of these vessels were filled with fibrin thrombi (Fig 3 B)



Fig 2

Microscopical view of renal cortical infarct The urface of the kidney is seen near the upper margin The subcapsular area is a site of heavy infiltration of inflamma tory cells whereas the central zone of the infarct (lower half) is acellular Van Gieson × 83

In non necrotic areas of the cortex there were extensive epithelial lesions in the proximal as well as in the distal convoluted tubules and in the loops of Henle Apart from disintegration and desquamation (features that may in part be due to autolysis particularly in the proximal convoluted tubules) the epithelium dis played prominent nuclear and cytoplasmatic pleomorphism There were hyper chromatic nuclei and rather frequent mitotic figures indicating regeneration (Fig 4 B) The disintegrated epithelium was often mixed with hyaline or granular casts in the lumina particularly in the collecting tubules of the medullary rays of the cortex (Fig 4 A) Most glomeruli in non necrotic areas were normal but in a few the epithelium of B wman s capsule vas swollen (Fig 4 A)

The medulla was a site of epithelial lesions similar to those in the cortex but less pronounced Many of the collecting tubules were filled with hyaline or granular casts Slight inte stitial infiltration of inflammatory cells mainly lymphocytes was present in non ne rotic areas of the cortex and in the medulla



Fig 3

A Lower margin of renal cortical infarct with prominent deposition of granular material PAS × 150

B Mural oedema and necrosis and fibrin thrombus in interlobar artery (left) at the margin of renal infarct Ladewig × 150

The liver was a site of moderate fatty degeneration The spleen had prominent hyaline changes in the arterial walls The pancreas and myocardium were normal

COMMENT

The clinical pattern displayed by this patient including intestinal symptoms developing within 24 hours after the consumption of tartaric acid definitely favours the diagnosis of poisoning (1 8) So do the widespread tubular lesions in the kidney (11) which are similar to those of experimental tartrate nephritis in dogs and rabbits described early in this century These experimentally produced lesions were said to consist of necrosis of the epithelium of the convoluted tubules and of the loops of Henle The glomeruli however were reported to be largely unaffected apart from the accumulation of "albuminous precipitate" (5) or large amounts of serum (9) in Bowman s capsule In our patient there was also a vascular component with oedema and necrosis of arterial walls and fibrin thrombi The mechanism of development of these vascular lesions is obscure Since there was no clinical history of hypertension or any histologic evidence of systematic vascular disease



Fig 4

A Hyaline casts and disintegration of the epithelium in collecting tubule (left) Some swelling of the epithelium of Bowman s capsule (right upper) Van Gieson × 150

B Irregular disintegrated tubular epithelium together with hyperchromatic nuclei and mitotic figures (arrows) indicating regeneration Van Gieson × 300

the intrarenal vascular changes are most probably related to the other renal lesions present It is difficult to assess however whether they were the cause of the cortical infarcts or developed secondarily

The nature of the granular material at the margin of the infarcts also remains to be explained In PAS stained sections the granules appeared intensely violet stained Though far from specific this staining reaction suggests the presence of tartaric acid salts (4)

Chemical analysis of kidney tissue was not performed since only formalin fixed material was available

SUMMARY

Fatal acute renal disease developed after the ingestion of approximately 30 g of tartaric acid The renal lesions consisted of cortical infarcts with fibrinoid necrosis and thrombi in the small intrarenal arteries and extensive degeneration of the tubular epithelium This latter feature corresponds to the previously described experimental tartrate nephritis in the dog and in the rabbit

REFERENCES

1. *Arena J* Poisoning Charles C Thomas Publisher Springfield Illinois 1963 p 251 p 306
2. *Dreisbach R H* Handbook of poisoning Lange Medical Publications Los Altos California 1963 4th ed p 146
3. *Goodman L* & *Gilman A* The pharmacological basis of therapeutics MacMillan New York 1965 3rd ed p 818
4. *Gurr E* A practical manual of medical and biological staining techniques Leonard Hill London 1953 pp 139 140
5. *Karsner H T* The vascular reactions in experimental acute tartrate nephritis J Pharm Exp Ther *9* 493 495 1917
6. *Locket S* Clinical toxicology Henry Kimpton London 1957 p 531
7. The Merck Index New Jersey 1960 7th ed p 1012
8. *Oettingen W F von* Poisoning Saunders 1958 2nd ed p 556
9. *Pearce R M* & *Ringer A I* A study of experimental nephritis caused by the salts of tartaric acid J Med Res *29* 57 64 1913
10. *Post W E* The effect of tartrates on the human kidney J Am Med Ass *62* 592 593 1914
11. *Sollmann T* A manual of pharmacology Saunders 1957 8th ed p 1025
12. *Underhill F P*, *Wells H C* & *Goldschmidt S* Tartrate nephritis with especial reference to some of the conditions under which it may be produced J Exper Med *18* 322 336 1913
13. *Underhill F P*, *Wells H C* & *Goldschmidt S* A note on the fate of tartrates in the body J Exper Med *18* 317–321 1913

Acta path microbiol scandinav 74 311-324 1968

The Departments of Oral Histopathology Periodontology and Oral Surgery
University of Umeå Umeå 6 Sweden

EPIDERMOLYSIS BULLOSA HEREDITARIA

5 *The Ultrastructure of Oral Mucosa and Skin in Four Cases of the Letalis Form*

By

TORE ARWILL AXEL BERGENHOLTZ and
HOLGER THILANDER

Received 19 ix 68

The first studies on the ultrastructure of the skin in epidermolysis bullosa hereditaria (e b h) were performed by *Pearson & Spargo* (1961) followed by *Pearson* (1962 1964) The simplex dystrophic and lethal types were studied In the *simplex* form there was a disintegration of the cytoplasm of the basal cells with no changes in the dermis In the *dystrophic* form the main symptom was a necrosis of the collagen of the upper dermis layer The basement membrane was largely intact and formed the roof in the bulla In the *letalis* case there was a separation between the basal cell layer and the basement membrane in the intermediate zone

Other investigators (*Lapiere, Castermanns Elias & Firket* 1964 *Vogel & Schnyder* 1965 1967) have criticized Pearson s classification *Lapiere et al* (1964) claim that their letalis case is not quite analogous to Pearson s single letalis case since in their case the changes are located primarily in the cytoplasm of the juxta dermal basal cells In two letalis cases *Bellone Caputo & Clementi* (1965) noted damage in the cytoplasm of the cells in the Malpighian layer In two of the three dystrophic cases studied by *Vogel & Schnyder* (1965 1967) and in one case studied by *Kobayasi* (1967) a separation was found in the same place as in Pearson s letalis case

As the interpretation of the morphological picture in the skin on an ultrastructural level is somewhat controversial it is of interest to investigate the problem further especially as ultrastructural changes in the oral tissues from epidermolysis bullosa hereditaria letalis (e b h l) have not been reported earlier in the available literature

MATERIAL AND METHODS

In four cases of e b h l (cases 8 9 10 and 11 in paper I of this series *Bergenholtz & Olsson* 1968) and in one three month old control case (operation material from a case with a cleft lip otherwise healthy) biopsies from clinically intact skin (forearm shoulder and lip) and oral mucosa (gingiva tongue and buccal mucosa)



Fig 1

Control case Gingiva Tonofilaments rather scanty Mitochondria and hemi desmosomes numerous

Fig 2

F b h 1 Mitochondria numerous hemi desmosomes and tonofilaments sparse A Gingiva B Skin


2

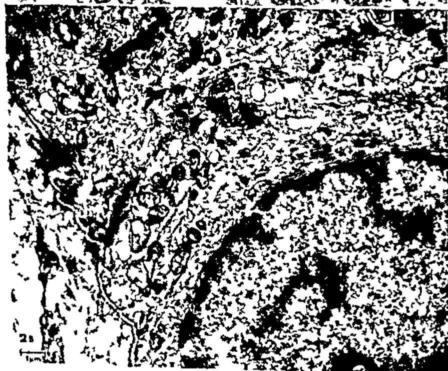

2a



Fig 3

Labial Gingiva. The epidermo dermal junction intact. In the connective tissue areas of oedema

were taken by excision with a scalpel and careful ... cut ... ut using punch or forceps in order to counteract blister form ... the specimens into one mm³ pieces with a razor blade the ... a chilled one per cent osmium tetroxide solution (*Rhodin* 1I

After embedding in epon the specimens were ... ultramicrotome. Post staining was performed with uranyl acetate ... Before thin sectioning survey sections were stained in to ... in a light microscope for orientation. A Zeiss electron mi... ...iskop II were used for viewing.

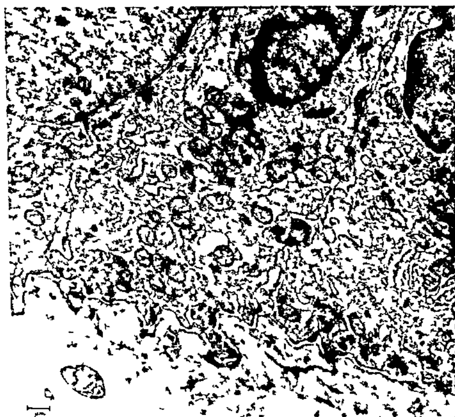


Fig 4

F b h 1 Gingiva Mitochondria numerous Beginning disintegration of some mitochondria The epidermal dermal junction intact Ordinary width of the intermediate zone

OBSERVATIONS

Control Case

Biopsies of skin (upper lip) gingiva (right upper second deciduous molar region) and oral mucosa (left bucca) were taken from a three month old child

It was noted that the sub epithelial connective tissue in the child is looser than in the adult The number of tonofilaments in the epidermis is reduced The epidermo dermal junction displays rather frequent hemidesmosomes The mitochondria in the basal epithelial cells are numerous (Fig 1)

Epidermolysis Bullosa Hereditaria Letalis Cases

In areas with an intact basement membrane it was noted that the epithelial tonofilaments are few in number The hemi desmosomes



Fig 3

Fbb 1 Gingiva The epidermo dermal junction intact In the connective tissue areas of oedema

were taken by excision with a scalpel and carefully lifted out with ut u ing punch or forceps in order to counteract blister formation After dividing the specimens into one mm³ pieces with a razor blade the specimens were fixed in chilled one per cent osmium tetroxide solution (Rhodin 1954)

After embedding in epon the specimens were sectioned in an LKB Ultrotome Post staining was performed with uranyl acetate and lead citrate Before thin sectioning survey sections were stained in toluidine blue and viewed in a light microscope for orientation A Zeiss electron microscope and a Siemens Elmiskop II were used for viewing



Fig 6

E.b h l Gingi a Beginning d sintegration in the cytoplasm of an epithelial cell forming a perinuclear halo zone Scattered mitochondria and tonofilaments in the zone

membrane could be seen (Fig 7) The structure of the hemi desmosomes appeared to be changed The ordinary lamellated pattern could not be discerned only a diffuse granular substance (Figs 8 A and B)

In some specimens a widening of the intercellular spaces and a marked increase in the size of the blisters were observed On the con nective tissue side oedema and free erythrocytes could be seen in the injured area Disintegration of cellular and intercellular components



Fig 7
Fbhl Gingiva Two blister formations in their initial stage The blisters form in the intermediate zone

was frequently present The cellular membranes were injured and the basement membrane was indistinct (Figs 9 and 10) The desmosomes appeared to be intact even in areas with widened intercellular spaces (Fig 11) In a few specimens there was a more or less total disintegration of the epithelium with a decrease in number of the desmosomes (*Fig 12*) *In the connective tissue adjacent to the blister there was a* lysis of the different structures

DISCUSSION

The specimens were taken as mentioned above from clinically intact skin and oral mucosa However as the children were greatly injured only small areas of intact skin and oral mucosa were found in some cases The observed changes could therefore be explained either as being present at the biopsy moment though the biopsy region looked clinically sound or experimentally produced in spite of the careful biopsy technique In our opinion most of the changes may depend on



Fig 8

A Control case Gingiva The hemi desmosomes have a lamellated pattern — B E b h l Gingiva No lamellated pattern can be observed in the area for the hemi desmosomes only a diffuse granular substance

the latter alternative as it is well known that minor trauma may cause the typical lesions of this disease and we have also found that ordinary punch biopsies almost invariably produce blister formation A careful biopsy technique therefore is essential in order to study initial changes of e b h l With our scalpel technique many of our specimens also seemed to be traumatically intact even at an ultrastructural level

In earlier light microscope investigations of cases of e b h l a separation at the epidermo dermal junction has been noted

The toluidine blue stained survey sections exhibited a picture consistent with earlier reports given for skin (*e g Roberts et al* 1960) and for oral mucosa (*Arwill Bergenholtz & Olsson* 1965) to which the reader is referred



Fig 9

Abb 1 Gingiva Beginning disintegration of epithelial cell membrane with evidence of injury and in some areas loss

Consistent with the observations by *Pearson* (1962) by *Vogel & Schnyder* (1965 1967) and by *Ritenfeld* (1966) oedematous changes in the subepithelial connective tissue were found (Fig 3) In our opinion and in agreement with *Kobayasi* (1967) these changes are an early sign in the pathogenesis concomitant with the initial disintegration of the basal cell mitochondria (Fig 4) It was not possible to determine whether one of these changes preceded the other

The halo formation around the basal cell nuclei eventually formed a zone of disintegration containing remnants of mitochondria and tonofilaments (Fig 6) The significance of this change is not readily apparent

In agreement with *Pearson* (1962) it was found that in a clinically intact area incipient changes could be noted in the form of the disappearance of the normal structure in the intermediate zone including the hemi desmosomes (Fig 8 B) The opinion that the basal cells rest directly against the basal membrane could not be verified however (Fig 4)

The structural changes in the intermediate zone could of course be of importance in the blister formation



Fig 10

E.b h l Gingiva Blister formation with erythrocytes At the connective tissue side of the blister the basement membrane is clearly seen

The observed changes are similar in all four cases and no differences between the pictures from the skin and those from different regions of the oral mucosa appear (gingiva bucca vestibulum oris and apex linguae)

The discrepancies in the observations published earlier may be ex



Fig 11
In the widened intercellular spaces the desmosomes appear intact.

plained by a confusion in the classification of the cases and possibly by a less adequate sampling technique

SUMMARY

In four cases of epidermolysis bullosa hereditaria letalis biopsies were taken from the skin and from different places in the oral mucosa (gin

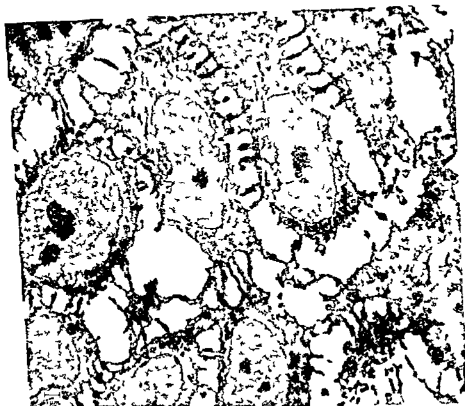


Fig 19

E b h l Gingiva Extreme widening of the intercellular spaces Some desmosomes can still be seen

giva bucca vestibulum oris and apex linguae) Controls were taken from a three month old healthy child

The electron microscopic observations in the e b h l cases were

(1) The tonofilaments and the hemi desmosomes were reduced in number
(2) The mitochondria were as numerous as in the control case
(3) Primary pathological changes were (a) oedema in the subepithelial connective tissue not far from the basement membrane (b) incipient disintegration of the mitochondria where the cristae disappeared in the basal cells as well as in the clear cells (c) an electron opaque perinuclear zone which eventually disintegrated leaving remnants of tonofibrils and mitochondria free (d) widened intercellular spaces between the epithelial basal cells
(4) The further development was characterized by the following changes (a) accentuated widening of the intercellular spaces but still with intact desmosomes (b) formation of minute vesicles between the basement membrane and the basal cell membrane in

the intermediate zone (c) disappearance of the lamellated pattern of the hemidesmosomes and substitution with a granular substance

(5) Late changes were (a) progression of the widening of the intercellular spaces (b) marked increase in the size of the blisters (c) injury to the cell membranes (d) increase in the oedema of the connective tissue and free erythrocytes indicating an injury to the capillaries

(6) Ultimately total disintegration of the epidermis and the underlying injured parts of the connective tissue resulted

(7) In all four cases the observations in the skin and the oral mucosa were identical

REFERENCES

Arwill T., Bergenholtz A & Olsson O Epidermolysis bullosa hereditaria IV Histologic changes of the oral mucosa in the polydysplastic dystrophic and letalis forms Odont Revy *16* 101–111 1965

Bellone A C, Caputo R & Clementi F La formazione della bolle nella epidermolisi bollosa congenita grave Osservasioni al microscopio elettronico Giorn Ital Derm *106* 135–146 1965

Bergenholtz A & Olsson O Epidermolysis bullosa hereditaria I Epidermolysis bullosa hereditaria letalis A survey of the literature and report of 11 cases. Acta dermat venerol *48* 230 241 1968

Kobayasi T Dermo epidermal junction in the recessive type of epidermolysis bullosa An electron microscope study Acta dermat venerol *47* 57–59 1967

Lapière S Castermans Elias S & Firket H Elektronenmikroskopische Untersuchungen über die Ultrastruktur des Epidermolysis bullosa letalis bei einem Säugling mit familiärer Belastung Hautarzt *15* 30–33 1964

Pearson R W Studies on the pathogenesis of epidermolysis bullosa J Invest Derm *38* 551 570 1962

Pearson R W Some observations on epidermolysis bullosa and experimental blisters The Epidermis L I Montagna W & Lobitz W C Jr Academic Press New York & London pp 613–626 1964

Pearson R W & Spargo B Electron microscope studies of dermal epidermal separation in human skin J Invest. Derm 36 213–224 1961

Rhodin J Correlation of ultrastructural organization and function in normal and experimental changed proximal convoluted tubule cells of the mouse kidney Thesis Stockholm 1964

Rittenfeld I Zur Histogenese und Differentialdiagnose hereditärer Epidermolysen Arch klin exp Dermat 224 128–137 1966

Roberts M H Howell D R S Bramhall J L & Reubner B Epidermolysis bullosa letalis Report of three cases with particular reference to the histopathology of the skin Pediatrics 25 283–290 1960

Vogel A & Schnyder U W Elektronenmikroskopische Untersuchungen an der rezessiven Epidermolysis bullosa dystrophica (vorläufige Mitteilung) Dermatologica *131* 81–89 1965

Vogel A & Schnyder U W Feinstrukturelle Untersuchungen an rezessiv dystrophischer Epidermolysis bullosa hereditaria Dermatologica *135* 149–172 1967

Acta path microbiol scandinav 74 325-332 1968

The University Institute for Experimental Medicine (Head Jens Bing M D)
71 Nørre Alle Copenhagen Ø Denmark

PENETRATION OF CIRCULATING FLUORESCENT PROTEINS INTO WALLS OF ARTERIOLES AND VENULES IN RATS WITH INTERMITTENT ACUTE ANGIOTENSIN HYPERTENSION

By

FINN OLSEN

Received 22 ii 68

The pathogenesis of hypertensive vascular disease has been studied in model experiments in various species Thus it has been observed *in vivo* in renal hypertensive rats through a window placed in the cranium that cerebral arteries reacted with local dilatations and constrictions along their courses The same patterns of response of vessels were observed along the arterioles situated at the surface of the small intestine (*Byrom* 1954) Further it has been shown that in acute hypertensive rats a deposition of intravenously injected fluorescent proteins takes place in the walls of arterioles in the pancreas intestine mesentery and heart (*Giese* 1961)

In addition it has been demonstrated in acutely hypertensive rats that the dilatations which *Byrom* (1954) observed were permeable sometimes for intravenously injected colloidal carbon particles while the constrictions were never permeable for such particles (*Giese* 1964) On basis of these observations Giese concluded that plasma proteins might accompany the colloidal carbon particles and settle in the wall of the vessel

The aim of this work has been to prove or to disprove Giese s assumption that plasma proteins just like colloidal carbon particles only settle in the arteriolar dilatations of acute hypertensive rats

MATERIAL AND METHODS

Animals White female rats weighing 180–200 grams were used

Anaesthesia A solution of amythal® 25 mg/ml was injected intraperitoneally at the rate of ¼ ml per 100 grams of rat

Fluorescent proteins A modification of Nairn s (1962) procedure using Lissamine Rhodamine RB 200 was employed. 250 mg of RB 200 were ground together with

This work was supported by grants from *Kong Christian X s fond* and the grant of Ebba Celinder

The author is indebted to Dr *L T Mann* for reading the manuscript.

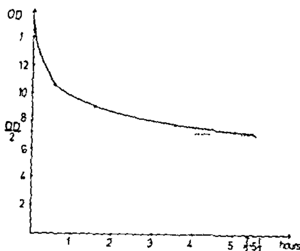


Fig 1
Rate of clearance from the plasma of a rat of homologous serum proteins conjugated with RB 200 The molecular ratio of fluoro brome to protein is about 1 7

500 mg of PCl_5 in a mortar for about five minutes and the mortar was as completely freed of the product (RB 200 converted to a sulphonyl chloride) as possible The recovered powder (about 300 mg) was added to about 125 mg porcine gamma globulin or homologous serum proteins in the course of half an hour at 0 C Unbound dye was removed partly with activated carbon and completely by filtration through a G 25 Sephadex column The molecular ratio of fluorochrome to protein ranged from 1 1 to 3 1 After this the half life of the conjugate in the circulation of a rat was measured in a spectrophotometer in the first hours after intravenous injection and found to be between 5½ and 7 hours (Fig 1) Schiller et al (1953) found the same half life for fluorescein labelled bovine serum albumin in the first hours after intravenous injection to rats

In each experiment 60–70 mg RB 200 labelled proteins were injected intravenously in a volume of about 2 5 ml of phosphate buffered physiological saline at pH 7 2 In response to the injection which was done in the course of two minutes the blood pressure rose 5–7 mmHg but decreased again to the normal value before the injection was completed Three rats were injected with RB 200 labelled homologous serum proteins and three rats with RB 200 labelled porcine gamma globulin

Angiotensin Hypertensin CIBA was dissolved in physiological saline

The technique of the experiments After complete anaesthesia had been induced tracheotomy was performed and the common carotid artery was cannulated with a needle for recording of the blood pressure The needle was connected to an aneroid manometer via a tube of polyethylene For intravenous injection of angiotensin and fluorescent proteins catheters of polyethylene were placed in the jugular vein and the femoral vein On account of the development of undesirable tachyphylaxia during continuous infusion of angiotensin repeated injections (½–1 microgram) were given with 4 5 minutes intervals between the injections This regimen caused the blood pressure to rise from about 90 mmHg to about 150 mmHg a rise which was known to produce the desired patterns of the vessels from the first series of injections Such experiments were fitted for the purpose because dilatations and constrictions can be reproduced exactly in the same places along the arterioles if they disappear in relation to the decrease of the blood pressure between the injections In this paper we define a dilatation as a local expansion of an arteriole the diameter of which is undoubtedly increased with respect to the diameter of the arteriole before the injection of angiotensin A constriction is defined as a local narrowing the diameter of which is undoubtedly smaller than the diameter of the arteriole before the injection of angiotensin The experiments lasted 2½ hours At the end of the experiment a window of plexiglass was placed in the abdominal wall of the rat In this way it was possible to observe a loop of the small intestine through a stereo

microscope and observe the patterns along the arterioles Then in relation to a rise of the blood pressure such a loop of intestine was frozen instantaneously in vivo with isopentane cooled to the freezing point with liquid nitrogen The window was not placed in the abdominal wall until at the end of the experiment because it was observed that the small intestine became quickly paralyzed and distended if the window had been in place throughout the entire 2½ hours duration of the experiment In earlier experiments with colloidal carbon particles as tracer for plasma proteins it was observed that light drying of the surface of the intestine and mechanical influents on the intestine changed the permeability of the vessels These faults were avoided using the described technique After the instantaneous fixation with isopentane the intestinal loop was fixed further in absolute alcohol in 6–8 days at minus 20 degrees centigrade Using this procedure the dilatations and the constrictions were preserved unchanged (Giese 1964)

Control animals These animals were treated in the same way as described in the technique of the experiments but were injected intravenously with physiological saline instead of angiotensin Three rats were injected with RB 200 labelled homologous serum proteins and one rat with RB 200 labelled porcine gamma globulin

Microscopical preparations After fixation the intestinal loop was cleared in methyl benzoate and dilatations and constrictions were dissected out under a stereomicroscope These were embedded in paraffin cut into five micron thick serial sections and finally examined under a fluorescent microscope

Some of the arterioles containing fluorescent proteins in the wall were stained later with periodic acid—Schiff's reagent (PAS) or with hematoxylin—eosin

RESULTS

The arterioles and venules towards which the attention was directed were 60–100 microns in diameter measured *in vivo* During periods of dilatation the diameters of the arterioles were increased by about 50 per cent in relation to the diameters before the injection of angiotensin The diameter of the constrictions was decreased by about 25 per cent

Dilatations From the three rats injected with RB 200 labelled homologous serum proteins 39 dilatations were examined by microscopy In 9 of these (about 23 per cent) fluorescent proteins were observed in the wall

From the three rats injected with RB 200 labelled porcine gamma globulin 29 dilatations were examined by microscopy In 5 of these (about 17 per cent) fluorescent proteins were observed in the wall

In the dilatations containing no fluorescent proteins in the wall it was noticed that the walls were very thin nearly like the walls of the venules

By serial sections it was possible to follow that the deposition of the fluorescent proteins were situated in a small part of the circumference of the wall in the first sections reached the largest extent in the following sections and then decreased again in extent until the wall contained no more fluorescent proteins (Fig 2)

Sometimes it was impossible to determine if the fluorescent proteins were situated partly along the luminal side of the endothelium and partly in the wall of the vessel Therefore some sections were stained with hematoxylin and eosin and examined by light microscopy In this way it was possible to determine the exact thickness of the wall It was found that the thickness of the wall in the section stained with hematoxylin and eosin in the majority of cases corresponded completely to

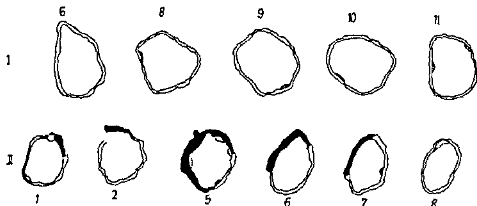


Fig 2

The superior horizontal row Illustrations of serial sections of a dilatation without depositing of fluorescent proteins in the wall The thickness of the wall is nearly the *same the wall around and from one section to another The inferior horizontal* row Illustrations of serial sections of a dilatation with depositing of fluorescent proteins in the wall The wall is thicker corresponding to the location of the fluorescent proteins than where no fluorescent proteins are deposited The illustrations are produced as a tracing from photographs of the dilatations stained with hematoxylin and eosin The nuclei are hatched and the deposition of the fluorescent proteins is painted black Magnification 306 ×



Fig 3

A Photographs of a dilatation with deposition of fluorescent proteins in the wall corresponding to tracing number one and five in the inferior horizontal row of Fig 2 B Photograph of a dilatation with depositing of fluorescent proteins in the wall in a hematoxylin eosin staining The photograph corresponds to the tracing number one in the inferior horizontal row of Fig 2 and to photograph number one in *Fig 3 A Magnification 680* ×

the width of the fluorescent proteins measured in the fluorescent microscope Only in very few places around the circumference of the wall a very small difference was measured between the thickness of the wall in the hematoxylin—eosin staining and the width of the fluorescent proteins *Corresponding* to these places it was to expect that a very thin border of fluorescent proteins was situated at the surface of intima In this way it was possible to conclude that the wall of the dilatation

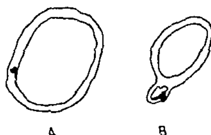


Fig 4

A Drawing of a grain of fluorescent protein in the wall of a constriction Magnification 340 × B Drawing of a branching of a constricted arteriole with penetration of fluorescent proteins into the wall of the leaving branch Magnification 340 ×

really was thicker corresponding to the position of the fluorescent proteins than that part of the wall which contained no deposition of fluorescent proteins (Figs 2 and 3)

When stained with PAS dilatations containing fluorescent proteins in the wall showed weak but undoubtedly positive PAS staining exactly where the fluorescent proteins were situated

Constrictions From the six rats 47 constrictions were examined by microscopy In none of these were found such marked depositions of fluorescent proteins in the wall as in dilatations but occasionally a grain of fluorescent protein was observed in the wall of a constriction On the other hand the endothelium was clearly permeable for fluorescent proteins corresponding to a branching of a constricted arteriole but this phenomenon was found in only one section (Fig 4)

Venules In the walls of the venules fluorescent proteins were recognized very often as a spotted intrusion (Fig 5) in spite of the fact that the venules were not reacting with the pattern of local dilatations and constrictions which was characteristic for the arterioles when angiotensin was injected intravenously A more uniform dilatation of the



Fig 5

Photograph of a part of a venule with location of fluorescent proteins partly at the luminal side of the intima and partly in the wall Magnification 680 ×

venules did not occur in relation to the injection of angiotensin. On the contrary it could be seen through the abdominal window that constriction of venules corresponded to the rise of the blood pressure following the intravenous injection of angiotensin and that they slowly returned to the state prior to the injection of angiotensin with an accompanying decrease in blood pressure.

Control animals. 10 arterioles and venules were examined by microscopy but in neither the arterioles nor the venules were fluorescent proteins recognized in the wall. Very often it was seen that the fluorescent proteins were situated as a thin border upon the luminal side of intima.

DISCUSSION

The named results confirm the assumption upon which *Giese* (1964) based his interpretation of results with colloidal carbon particles that plasma proteins mainly deposit in the wall of dilatations of the arterioles in acute hypertensive rats. But besides the localization in these areas the fluorescent proteins have disclosed that in constrictions the endothelium was in some areas permeable for a "grain" of fluorescent protein. Corresponding to a branching of a constricted arteriole it was found in one section that the endothelium was permeable for fluorescent proteins.

On basis of these results it has been established that dilatations account in a considerable degree for a depositing of fluorescent proteins in the walls of arterioles. In this way dilatations are a very important structural change in the arterioles in the analysis of the pathology of the acute hypertensive damage of the vessels in rats.

Because fluorescent proteins have been recognized in the walls of dilatations in only about 20 per cent of all such areas that have been examined under the fluorescent microscope it may be concluded that dilatation is not alone sufficient to account for the deposition of fluorescent proteins in the wall of the arteriole. An additional factor must be important before plasma proteins will penetrate into the wall of the dilated arterioles. A similar view was taken by *Giese* (1966) in his thesis (page 115–116) based in part upon *Abt & Bruckner*'s (1960) and *Byrom*'s (1963 a) negative histological findings in examination of vessels from the retinas of hypertensive rats containing dilatations and constrictions and in part upon the results of the technique which *Giese* (1964) used with colloidal carbon particles as tracer for plasma proteins. By stereo microscopy he observed many dilatations in a single field of vision but only very few of these contained colloidal carbon particles in the wall.

Because colloidal carbon particles are phagocytized by cells belonging to the reticulo-endothelial system in the course of 10 minutes after the intravenous injection it is possible that other cells (for example endothelial cells) handle the colloidal carbon particles in a way different

from plasma proteins Therefore it has been desirable to use fluorescent proteins in order to compare the results obtained with colloidal carbon particles In addition the fluorescent proteins give a better quantitative impression of the permeability of the endothelium in dilatations than colloidal carbon particles on account of the longer persistence of the fluorescent proteins in the circulation

In 1958 *Duguid & Anderson* published histological findings from 72 cases of hypertensive nephrosclerosis These results indicated that the hyaline thickening which can be observed in the arterioles of hypertensive patients could be produced first by a deposition of components from plasma upon the luminal side of the endothelium and second by a growth of endothelial cells across the deposit of the plasma components In the present study of acute hypertensive experiments with rats it has not been possible to observe such a development of the hyaline thickening of arteriolar walls On the contrary it has clearly been observed that the walls of the arterioles were thicker in the places where fluorescent proteins had penetrated the endothelium than in places where no fluorescent proteins had been deposited in the wall The deposition of plasma proteins in the walls of the arterioles had a hyaline appearance when stained with hematoxylin and eosin The nuclei of the smooth muscle cells were normal

SUMMARY

In rats with intermittent acute hypertension after repeated intravenous injections of large doses of synthetic angiotensin dilatations along arterioles situated at the surface of the small intestine were disposed for the penetration of circulating fluorescent proteins into the arteriolar wall That part of the wall where the fluorescent proteins were situated was thicker than that part of the wall where no deposition of fluorescent proteins was observed In some places in the constrictions the endothelium was permeable for a grain of fluorescent protein Corresponding to a branching of a constricted arteriole it was found in one section that the endothelium was permeable for fluorescent *proteins* Because it has been possible to observe fluorescent proteins in the walls of dilatations only in about 20 per cent of the areas which were examined in the microscope we conclude that besides dilatation itself an additional factor is required before plasma proteins will penetrate the walls of the *dilated* arterioles It was observed that the endothelium of the venules was permeable for fluorescent proteins in spite of the fact that venules did not react with dilatations during the intravenous injection of angiotensin

REFERENCES

1 Abt K & *Brückner R* Netzhautgefassspasmen bei artifiziell hypertonischen Ratten Ophthalmologica (Basel) *119* 17–43 1950

2 *Byrom F B* The pathogenesis of hypertensive encephalopathy and its relation to the malignant phase of hypertension Experimental evidence from the hypertensive rat Lancet *II* 201–211 1954
3 *Byrom F B* The nature of malignancy in hypertensive disease Evidence from the retina of the rat Lancet *I* 516–520 1963 a
4 *Duguid J B* & *Anderson G S* The pathogenesis of hyaline arteriolosclerosis J Path Bact *64* 519 532 1952
5 *Giese J* Deposition of serum proteins in vascular walls during acute hypertension Acta path microbiol scandinav *53* 167–172 1961
6 *Giese J* Acute hypertensive vascular disease 2 Acta path microbiol scandinav *62* 497 515 1964
7 *Giese J* The pathogenesis of hypertensive vascular disease Munksgaard Copenhagen 1966
8 *Nairn R C* Fluorescent protein tracing Edinburgh & London 1962
9 *Schiller A A* *Schayer R W* & *Hess E L* Fluorescein conjugated bovine albumin—physical and biological properties J gen Physiol *36* 489–506 1953

Acta path microbiol scandinav 74 333-339 1968

The Department of Hygiene and the Neuropathological Laboratory
Department of Pathology I University of Goteborg Sweden

ARTEFACTITIOUS EXTRAVASATION OF FLUORESCENT INDICATORS IN THE INVESTIGATION OF VASCULAR PERMEABILITY IN BRAIN AND SPINAL CORD

By

LENNART RINDER

Received 23 iv 68

An increase of vascular permeability with exudation of plasma proteins is a common accompaniment of many diseases and injuries of the central nervous system Owing to the role played by such changes in the development of cerebral oedema a wider knowledge of cerebro spinal vascular permeability in various conditions is of considerable importance (c f *Bakay & Lee* 1965 *Klatzo* 1967)

In recent investigations vascular permeability has often been studied for changes by examination of the microscopic distribution of fluorochrome or radioactive labelled serum proteins in thin sections (c f *Klatzo et al* 1962 *Bakay & Lee* 1965 *Klatzo* 1967) These indicators are considered normally not to penetrate the cerebral blood vessels except in some specialized areas such as the choroid plexus tuber cinereum the hypophyseal stalk and area postrema (c f *Klatzo et al* 1962 *Klatzo & Steinwall* 1965) —While studying vascular permeability changes following experimental brain concussion (*Rinder & Olsson* 1968) extravasation of fluorochrome labelled albumin was sometimes observed in the brain stem and spinal cord also in control animals Most previous investigations with similar permeability indicators have been confined to the cerebral hemispheres It is possible that unpredictable microscopic artefactitious extravasation may be hard to avoid in certain parts of the brain and spinal cord Clarification of this point is urgent for future microscopic studies of vascular permeability changes and the formation and migration of oedema in the brain and spinal cord

It was with this in mind that the present investigation was started in which the distribution of circulating fluorescent vascular permeability indicators in the brain and spinal cord was charted with various methods of removal fixation and preparation of the tissues

The investigation has been supported by grants from *St ftelsen VS fonden* and *Trygg Fylgias Attiodrsfond*

MATERIALS AND METHODS

25 rabbits (2.0–3.2 kg) and 8 Sprague Dawley rats (250–350 g) were used. During the injection of the indicator and at sacrifice the animals were under light anaesthesia: sodium mebumal[1] injected slowly i.v. in the rabbits and ether in the rats. Before injection of the indicator a polyethylene catheter was inserted into the abdominal aorta in the rabbits.

Permeability indicators. The following indicators were used:

1. Evans blue thoroughly mixed with a 5 per cent Ringer solution of bovine or rabbit serum albumin[2] to make a 1 per cent dye solution (EBA).

2. Bovine albumin[2] labelled with fluorescein isothiocyanate[3] according to *Klatzo et al.* 1962 (FLA).

Before being injected EBA was filtered and FLA passed through a Sephadex G 25[4] laboratory column.

A volume of 1 ml per 100 g bodyweight was injected i.v. or through the catheter in the femoral artery.

TABLE 1

Survey of Number of Animal Experiments. Methods of Fixation of Brain and Spinal Cord and Preparation of Microscopic Sections of Normal Animals that Had Received Intravascular Injection of EBA or FLA.

Fixation	Sectioning	Rabbits	Rats
Immersion fixation	Freeze microtome	8	2
	Paraffin embedding	2	–
In situ immersion fixation	Freeze microtome	2	2
Perfusion fixation	Freeze microtome	7	2
	Paraffin embedding	4	–
Perfusion (saline) + immersion fixation	Freeze microtome	2	2

Sacrifice, removal and fixation of tissues. The animals were sacrificed at varying intervals (1–4 hours) after the injection of the indicator by bleeding through the catheter or opening of the right part of the heart. The specimens to be studied were removed and fixed in the following ways (Table 1).

1. Immediately after the exsanguination the dorsal part of the skull bones and the spinal column were carefully removed. The spinal cord was divided at C 7, removed together with the brain and placed in 10 per cent formalin for a few minutes. With a razor blade 3–4 mm thick coronal slices were cut from the cerebral hemispheres and at several levels of the pons, medulla oblongata and spinal cord and placed in formalin for at least 24 hours (*Immersion fixation*).

2. The dorsal part of the skull bones and spinal column were removed in the way described. The neck was cut at the level of C 7 and the whole head and neck placed in 10 per cent formalin with the brain and cervical cord *in situ* for about 48 hours. The brain and cord were afterwards removed and cut coronally (*In situ immersion fixation*).

3. Heparin was injected i.v. and immediately afterwards the heart was opened and 250 ml saline perfused via the catheter in the abdominal aorta followed by 1000 ml of 10 per cent *formalin*. The perfusion pressure used was about 1.0 cm H$_2$O and the perfusion time about 30 minutes. The animals were left at room temperature for about 24 hours after which the brain and spinal cord were removed and cut in the way *described* above.—In the rats the catheter was inserted into

1 Mebumal® ACO Sweden.
2 Fluka AG Switzerland.
3 Nutritional Biochemicals Corp. U.S.A.
4 Pharmacia Fine Chemicals Sweden.



Fig 1
Section of immersion fixed specimen from cerebral hemisphere (rabbit) FLA confined to the lumen of the vessel



Fig 2
Section of immersion fixed specimen from the brain stem (rabbit) Extravasation of FLA Note uptake of FLA in cells

the aorta immediately after they had been sacrificed and the perfusion was performed with corresponding volumes of fluid per unit of bodyweight (*Perfusion fixation*)

4 In some rabbits 250 ml of saline was perfused and the brain and spinal cord removed immediately afterwards cut in the wa described above and fixed by immersion in formalin

Microscopic sections Sections 8–10 μ thick were cut on a freezing microtome and mounted in 50 per cent aqueous glycerine Sections were also cut from paraffin bl cks and mounted in Entellan 1

Microscopic examination The mounted sections were examined in a Zeiss fluorescence microscope equipped with dark field condenser and UV source of light Osram HBO 00 The light was filtered through a Scott BG 12/3 exciter filter and the emitted light passed through a filter combination of Scott OG 4 and OO 4 Microphotographs were taken on Ektachrome Agfa IF and Kodak Tri X films

EBA was recognized in the fluorescence microscope by its intense red fluorescence (*Steinwall & Klat o* 1965 *Hamberger & Hamberger* 1966) and FLA by its intense green fluorescence

Merck AG Germany

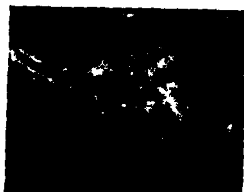




Fig 3 Fig 4

Fig 3 Section of immersion fixed specimen from the cervical spinal cord (rat) *Marked extravasation of FBA*

Fig 4 Section of perfusion fixed specimen from the brain stem (rabbit) No trace of the indicator (LBA)

RESULTS

The distribution of the fluorescent permeability indicators in the *sections varied considerably with the way in which the sections had* been obtained and fixed

In tissues removed immediately after sacrifice and fixed by *immersion* in formalin the indicators were confined to the lumina of the blood vessels in sections of the cerebral hemispheres (Fig 1) Pericytes containing yellow autofluorescent material facilitated recognition of the spread of the indicators (c f *Klatzo et al* 1962) In some parts of the sections of the brain stem and spinal cord the indicators were in most of the experiments observed both within and outside the walls of blood vessels (Figs 2 and 3) Indicator fluorescense was often ob*served within nerve and glial cells in these areas (Figs 2 and 3)* This fluorescense was always diffuse in the cytoplasm but often confined only to parts of the nucleus In animals killed more than 1 hour after the injection of EBA some cells of the ependymal lining of the ventricles and central canal were faintly red fluorescent

In the grey matter of the cervical cord extravasation was sometimes fairly extensive Exudation was never so extensive in the brain stem where it was mostly confined to the vicinity of the exit of the cranial nerves

Sections of specimens fixed by *immersion in situ* showed changes similar to those seen *in sections of specimens fixed by immersion in* formalin after removal of the brain

In the sections obtained from specimens rinsed with saline and fixed by *perfusion* with formalin and then left intact for about 24 hours the indicators were seldom observed in the lumina of the blood vessels which mostly appeared empty (Fig 4) Sometimes a thin fluorescent

zone outlined the inner surface of the endothelium but the indicator never extended significantly into or outside the vessel wall except in the area postrema where such an intramural and extramural ocurrence of the indicator was the rule In animals allowed to survive more than 1 hour a faint red fluorescense was sometimes observed in ependymal linings especially in the floor of the fourth ventricle —The walls of the blood vessels displayed a greenish autofluorescense and contained yellow fluorescent pericytes (Fig 4) Myelinated areas were bluish autofluorescent and the cellular elements were mostly greenish with granular cytoplasm and dark nuclei

When removed *en bloc* and cut in a fresh state after perfusion with saline (but not formalin) there was no extravasation of the indicator into the brain and spinal cord

No differences were found between the results of experiments performed with bovine and rabbit serum albumin

The red fluorescense of EBA in frozen sections was initially intense but diminished considerably in the course of a few days and the sections became intensely greenish fluorescent In the paraffin sections the intensity of the red fluorescense seemed to be comparable to that of the frozen sections and did not fade much in the course of a month

DISCUSSION

The present study revealed that extravasation of circulating fluorochrome labelled serum albumin may occur in the brain stem and spinal cord in normal animals after fixation of the brain *in situ* or after fixation of the carefully removed and cut tissues by immersion in formalin No extravasation of the indicators was observed in specimens when the cerebrospinal vessels had been rinsed with saline and fixed by intravascular perfusion with formalin *i e* the same result as that obtained within the cerebral hemispheres whether fixed by immersion or perfusion It therefore seems reasonable to assume that the brain does not differ fundamentally from the spinal cord regarding the permeability of the blood vessels to the indicators used

However an unpredictable microscopic extravasation can evidently occur in the brain stem and spinal cord in association with the removal of unfixed tissues It is difficult to determine the factors contributing to this extravasation with certainly because in experiments using perfusion fixation the indicator is rinsed from the circulation before removal of the brain *Klat o et al* (1964) suggest that hypoxia and other metabolic disturbances in connection with the sacrifice of the animal may affect the normal vascular permeability However *Broman* (1949) has shown that the brain may be perfused with Trypan blue after an interval of up to 1 hour from the time of the sacrifice of the animal without any macroscopic extravasation of the indicator —

Traumatization of the tissues in association with the removal and sectioning of the specimens may be of importance *Broman* (1950) found that mechanical traumatization during removal of the brain may lead to ruptures of small superficial vessels During such removal it is very hard to avoid contact with the brain stem and spinal cord and traction of nerve connections The importance of mechanical traumatic effects in producing extravasation of the permeability indicators in these areas is strongly suggested by the fact that no extravasation was observed when the cerebrospinal vessels were rinsed by perfusion with saline before the brain was removed and fixed by immersion in formalin It would thus appear that intravascular indicators escape into extravascular compartments in association with the handling of the unfixed tissues

An uptake of the fluorescent indicators was often observed in cells in areas of extravasation When studying the distribution of intracisternally injected labelled serum proteins *Klatzo et al* (1964) observed an uptake of indicator by individual axons and subpial astrocytes in the spinal cord though rarely in animals perfused with formalin As such changes have been regarded as reflecting injury of the cells (*Klatzo et al* 1962) the uptake of the indicator was believed to be an artefact produced either by terminal anoxia or trauma due to sectioning of the tissues in a fresh state *Gray* (1961) attributed the occurrence of dark cells in the cerebellum to pressure effects during the handling of the tissues

It was thought that effects of mechanical trauma might be considerably less severe if the brain and spinal cord were left *in situ* after opening of the skull and spinal column and fixed by immersion of the whole head and neck in formalin But it did not prove to be so in the present experiments However *Mehrein & Hollander* (1966) found that labelled albumin was extravasated when the tissue blocks from the brain fixed by immersion in formalin were too large to allow rapid fixation also of the interior of the specimens

SUMMARY

The cerebrospinal vascular permeability in normal rabbits and rats was studied by observing the microscopic distribution of fluorochrome labelled serum albumin injected intravenously or intra arterially The tissues were removed fixed and prepared by various methods The following observations and conclusions were made

1 In tissue specimens freshly removed cut and immersion fixed the indicators were sometimes observed within the walls of cerebrospinal blood vessels and extravascularly and in various cellular elements These changes were most prominent in the grey matter of the spinal cord but were also observed in the brain stem

2 No extravasation of the indicators was seen in tissues rinsed and fixed by perfusion with saline and formalin respectively The blood vessels of the spinal cord are thus probably not permeable to serum proteins and in this respect they do not differ from the blood vessels in the main part of the brain
3 Rinsing and fixation of the brain and spinal cord before removal with saline and formalin respectively appears to prevent artefactitious extravasation of the permeability indicators
4 Mechanical traumatization of the tissue specimens is probably one of the main causes of artefactitious extravasation of the indicators

REFERENCES

Bakay L & Lee J C Cerebral Edema C C. Thomas Publ Springfield Ill U S A 1965

Broman T The permeability of the cerebrospinal vessels in normal and pathological conditions Munksgaard Copenhagen 1949 p 28–31

Broman T Supravital analysis of disorders in the cerebrovascular permeability III A critical analysis of the technique and results obtained in experimental animals Acta psych neurol scand 25 19–31 1950

Gray E G The granule cells mossy synapses and Purkinje synapses of the cerebellum light and electron microscope observations J Anat 95 345–356 1961

Hamberger A & Hamberger B Uptake of catecholamines and penetration of trypan blue after blood brain barrier lesions Z Zellforsch 70 386–392 1966

Klatzo I Neuropathological aspects of brain edema J Neuropath Exp Neurol 26 1–14 1967

Klatzo I Miquel J & Otenasek R The application of fluorescein labelled serum proteins (FLSP) to the study of vascular permeability in the brain Acta Neuropath 2 144–160 1962

Klatzo I Miquel J Ferris P J & Prokop J D Observations on the passage of fluorescein labelled serum proteins (FLSP) from the cerebrospinal fluid J Neuropath Exp Neurol 23 18–35 1964

Klatzo I & Steinwall O Observations on cerebrospinal fluid pathways and behaviour of the blood brain barrier in sharks Acta Neuropath 5 175 1965

Mehren P & Holländer H Die Bedeutung der histologischen Vorbehandlung bei Untersuchungen der Blut Hirn Schranke mit fluorescenzmarkierten Proteinen Histochemie 6 131–137 1966

Rinder L & Olsson Y Vascular permeability changes in experimental brain concussion A preliminary report Acta path microbiol scandinav 72 350–352 1968

Steinwall O & Klatzo I Double tracer methods in studies on blood brain barrier dysfunction and brain edema Acta neurol scand 41 591–595 1965

Acta path microbiol scandinav 74 340–35? 1968

The Institute of Pathology, General Hospital, Malmö, and the Institute of Forensic Medicine, University of Lund

CANCER METASTASIS TO THE CIRRHOTIC LIVER

A Study of Experimental and Autopsy Material

By

THORBJORN BERGE and TORE SALDEEN

Received 25 iv 68

Experimental investigations have shown that tumour cells injected into the portal vein or the hepatic artery produce tumours more often in the cirrhotic than in the non-cirrhotic liver (*Fisher & Fisher* 1960, *Saldeen* 1963). It appears from some statistical analyses of human autopsy material that tumours metastasize more often to a non-cirrhotic liver (*Lisa et al* 1942, *Lieber* 1957, *Gall* 1960). This disagreement may be due to the differences between liver cirrhosis induced in animals and human liver cirrhosis, to the choice of less suitable methods in comparative analyses, or to both.

In the experimental investigations on record liver cirrhosis was induced in rats by administration of carbon tetrachloride. In such cirrhosis the connective tissue is situated particularly around the central veins, i.e. centrolobular cirrhosis. But the commonest type of human liver cirrhosis in man is the portal form. Therefore two groups of rats were studied, one with central cirrhosis (carbon tetrachloride induced) and one with portal cirrhosis (foreign protein cirrhosis, f. p. cirrhosis).

The frequency of hepatic metastasis in man with and without cirrhosis was studied in an autopsy material. In the statistical analyses primary tumours in the same sites and in the same stage of metastasis were compared.

MATERIAL AND METHODS

Experimental Investigation

Rous rat sarcoma cells (*Ahlstrom & Jonsson* 1962) were injected into the portal vein or under the liver capsule (*Saldeen* 1963) of female rats of the Sprague Dawley strain weighing about 175–200 grams. Tumour cell suspensions were prepared by a modification of the trypsin DNAase method described by *Madden & Burk* (1961). Viable tumour cells were counted in Buerker's haemocytometer according to Schrek (1936).

Liver cirrhosis was produced either by intramuscular injection of 0 [illegible] ml of [illegible] per cent carbon tetrachloride in mineral oil 3 times a week for 3 months, or by intravenous injection of 2 ml of skim milk 3 times a week for one month (f. p. cirrhosis).

Tumour cells were injected about 14 days after the last injection of carbon tetrachloride or skim milk. At autopsy the liver was cut into 1 mm thick slices and the tumours were counted. The volume of the tumours was calculated with Schrek's formula (1935) $V = 0.5236 \times d_1 \times d_2 \times d_3$, where d_1, d_2 and d_3 are the 3 perpendicular dimensions of the tumour.

Autopsy Material

The material consisted of 10 160 autopsies (including 865 stillborns or newborns) performed in the years 1958–1965 In Malmo (about 250 000 inhabitants in 1965) *there is only one institution of pathology and during these years* 58 2 per cent of all persons who died in the town were autopsied (about 95 per cent of all who had died at Malmo General Hospital)

Throughout the period all autopsies were performed in a standardized way The lungs myocardium kidneys liver and spleen were always examined *histologically* The procedure was otherwise modified according to the nature of the case and the microscopic examination was often extensive Only remote growths were accepted as metastases care being taken not to include continuous growth from the primary tumour or from metastases As a rule lymph nodes from the supraclavicular axillary and inguinal fossae mediastinum and retroperitoneal space were examined as well as the regional lymph nodes close to the primary tumour Secondaries found in any of the above lymph node stations were counted as a single metastasis wether the cancer had been demonstrated in one or in more lymph nodes in the same station

In all cases with liver cirrhosis several sections of the liver were examined Only cases of so called portal cirrhosis were included in the material Liver cirrhosis was *regarded as present only when all parts of the liver were damaged with regeneration* and formation of pseudolobules surrounded by connective tissue Since statistical analysis of mild and severe cirrhosis revealed no differences in respect of metastases the two groups were pooled

In the statistical analysis of the findings importance was attached to the *site* of the primary tumour and its stage of metastasis (number of organs and *lymph node* stations with secondary growth) Primary liver cancer was excluded

The calculations were performed with an electronic computer[1]

RESULTS

Experimental Investigation

Experiment 1 Thirty six rats were given 10^6 living tumour cells intraportally Twelve were controls 12 had CCl_4 induced cirrhosis and 12 had f p cirrhosis The rats were killed 14 days after the injection of the tumour cells and the tumour metastases in the liver were counted

TABLE 1

Metastasis in Experimental Cirrhosis

	Number of animals	Number of metastases	Bodyweight (g)	Adrenals (mg)	Hypophysis (mg)
Controls	12	4 ± 1	245 ± 12	57 ± 5	10 ± 0 1
F p cirrhosis	12	102 ± 11	240 ± 10	61 ± 9	12 ± 0 4
CCl_4 cirrhosis	12	75 ± 16	250 ± 16	56 ± 6	10 ± 0 3

The number of metastases in the rats with liver cirrhosis was significantly *higher* than in the controls (Table 1) The tumour metastases were as a rule somewhat smaller in the animals with f p cirrhosis (Fig 1) than in the animals with CCl_4 induced cirrhosis (*Fig 2*) *but* more numerous in the former ($0.1 > p > 0.05$) As regards bodyweight and weight *of* the adrenals or anterior lobe of the hypophysis no

[1] We thank fil kand *Lennart Bergryd* and fil kand *Arne Sundström* of the Computer Centre in Lund for help with this analysis



Fig 1
F p cirrhosis with metastases

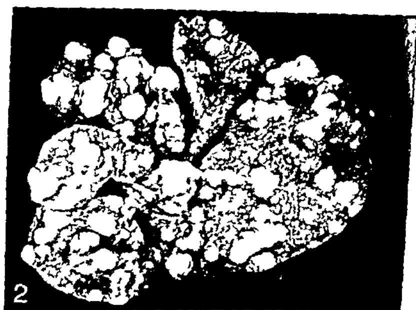


Fig 2
CCl_4 cirrhosis with metastases

differences between the different groups were found i e a finding arguing against a stress effect of the substances injected

Histologically the animals that had received carbon tetrachloride showed central cirrhosis of moderate severity

The animals that were given skim milk (cow) had monolobular cirrhosis of Laennec type The portal zones contained single eosinophilic leucocytes and plasma cells Heart lungs kidneys adrenals and spleen were microscopically normal

Experiment 2 Five control rats 6 with CCl_4 induced cirrhosis and 6 with f p cirrhosis were used All 17 animals were given 0 05 ml of a tumour suspension under the liver capsule The suspension contained 5×10^4 living tumour cells The animals were killed 14 days after the injection

Autopsy invariably revealed small rounded tumours at the site of the injection The size of the tumours was 70 ± 16 mm³ in the untreated rats 87 ± 17 mm³ in the rats with CCl_4 induced cirrhosis and 67 ± 27 mm³ in rats with f p cirrhosis The differences between the various groups were not significant

Human Series

A *Material* The sites of the primary tumours and the total number of metastasising tumours are given in Table 2

Out of 10 160 subjects autopsied 3 481 (34 3 per cent) had altogether 3 801 carcinomas 2 595 (68 3 per cent) of which had metastasized 385 (3 8 per cent) of the subjects had cirrhosis Out of the 9 775 subjects without liver cirrhosis 3 312 (33 9 per cent) had extrahepatic carcinoma (If the figure be corrected for the 865 stillborns and newborns the frequency will be 37 2 per cent) Out of the subjects with liver cirrhosis 93 (63 males and 30 females) or 24 2 per cent had extrahepatic carcinoma This sex distribution was the same as that of all subjects with cirrhosis

The average age of the subjects with cirrhosis was 68 2 years among those with carcinoma 70 1 years and among those with both cirrhosis and extrahepatic carcinoma 71 6 years

The figures given below refer to the number of *tumours* and not to the number of subjects with tumour There were 3 642 extrahepatic carcinomas 2 491 (68 4 per cent) of which had metastasized 108 of the tumours 57 (52 8 per cent) with metastases were seen in subjects with cirrhosis There were thus 3 534 carcinomas without coexisting cirrhosis Among these 2 434 (68 9 per cent) had metastasized Twenty three cases (21 3 per cent) in the cirrhosis group had liver metastases compared with 1 256 (35 5 per cent) in the group without liver cirrhosis This difference as measured with the χ^2 test was significant ($0\ 01 > p > 0\ 001$)

B *Analysis of results* The frequencies of liver metastasis found in subjects with and without liver cirrhosis are not strictly comparable

TABLE 2
Sites and Numbers of Primary Tumours

Site	Number of tumours			Number of tumours that had set up secondaries		
	female	*male*	*Total*	*female*	*male*	*Total*
Prostate	–	696	696	–	180	180
Lung	99	312	411	76	281	357
Stomach	171	224	395	148	191	339
Colon	188	166	354	128	100	228
Breast	330	2	332	310	2	312
Kidney	82	122	204	36	58	94
Pancreas	66	100	166	62	96	158
Liver	49	110	159	30	74	104
Bile ducts	98	51	149	87	43	130
Rectum	63	80	143	47	58	105
Ovary	131	–	131	119	–	119
Urinary bladder	34	81	115	22	53	75
Small intestine	38	56	94	11	15	26
Uterine cervix	82	–	82	70	–	70
Oesophagus	20	47	63	17	33	50
Thyroid	33	15	48	20	13	33
Skin	21	23	44	17	19	36
Corpus uteri	40		40	29	–	29
Renal pelvis + ureter	10	9	19	9	8	17
Vulva	12	–	12	10	–	10
Larynx	0	11	11		7	7
Appendix	5	6	11	1	3	4
Eye	4	5	9	4	5	9
Maxillary sinus	3	6	9	1	4	5
Oral cavity	3	6	9	2	5	7
Uterus (unspecified)	8		8	7	–	7
Hypopharynx	1	6	7	1	3	4
Anus	5	2	7	4	1	5
Tongue	3	3	6	2	1	3
Epipharynx	1	3	4	1	3	4
Salivary gland	2	2	4	2	1	3
Adrenal	2	2	4	2	2	4
Uterine tube	3	–	3	3	–	3
Lip	0	2	2	0		2
Tonsil	0	2	2	–	2	2
Vagina	2	–	2	1	–	1
Trachea	1	0	1	1	0	1
Penis	–	1	1	–	0	0
Cardia	1	0	1	1		1
Sweat gland	0	1	1	–	0	0
Testis	–	13	13		12	12
Unknown	22	17	39	[illegible]	17	39
Total	1633	2168	3801	1303	1292	2595

In such a comparison the following three factors must be taken into account

1. Frequency of metastasizing tumours
2. Stage of metastasis of the tumours
3. Sites of the primary tumours

Ad 1 Among the cases with and without liver cirrhosis 52.8 per cent resp. 68.9 per cent of the *carcinomas had metastasized* This presumably implies that also liver metastases are less liable to occur in patients with liver cirrhosis If only metastasizing tumours be included in the comparison non cirrhotic livers show metastases in 51.6 per cent (1 256 out of 2 434) and cirrhotic in 40.4 per cent (23 out of 57) The difference will then no longer be significant (0.1 > p > 0.05)

Ad 2 Among cases with metastases to *several* organs (advanced stage of metastasis) the expected frequency of metastases to a given organ should be higher than that among cases with *few* metastases (early stage of metastasis) This is clear from Table 3 which shows that the frequency of secondaries increases with the stage of metastasis to cirrhotic as well as to noncirrhotic livers

TABLE 3

Involvement of Liver in Subjects with Secondaries in 1 2 3 4 5 ≧ 6 Organs

Number of sites of metastasis	Cirrhosis		Not cirrhosis	
1	11.1 %	33.3 %	9.9 %	33.8 %
2	33.3 %		3[illegible].6 %	
3	50.0 %		5[illegible].5 %	
4	57.1 %		58.6 %	
5	100 %		73.[illegible] %	
≧ 6	100 %		83.[illegible] %	
	40.4 %		51.6 %	

Table 3 shows that tumours with secondaries at 1–4 sites set up metastases to the liver equally often in the presence as in the absence of cirrhosis Totally however the frequency is 10.2 *per cent higher in the group* without cirrhosis because of the greater number of tumours in an advanced stage of metastasis in this group

In an attempt further to elucidate the importance of the stage of *metastasis metastasizing tumours were divided into two groups* one with and one without secondaries in the liver (Figs 3 and 4)

Fig 3 shows that the frequencies of tumours in an early stage of metastasis (1–4 sites) is higher in the cirrhosis group In this group only 25 *per cent were* in an advanced stage of metastasis (> 4 sites) against 50 per cent in the group without cirrhosis Among these tumours in an advanced stage of metastasis all those with cirrhosis had metastasized to the liver but only 88 *per* cent of those without cirrhosis

In Fig 4 the tumours that had set up *only extrahepatic* secondaries are grouped in the same way The cirrhosis group included no tumours in an advanced stage of metastasis (more than 4 sites) Of the group

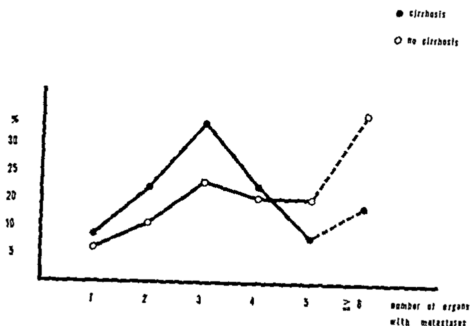


Fig 3
Frequency of different stages of metastasis in cases without liver metastases

%
50
45
40
35
30
25
20
15
10
5

● cirrhosis
○ no cirrhosis

1 2 3 4 5 ≧ 6 number of organs with metastases

Fig 4
Frequency of different stages of metastasis in cases without liver metastases

without cirrhosis however about 12 per cent had metastasized to more than 4 sites but not to the liver

Ad 3 As regards the tumours drained by vena portae the first capillary net is in the liver These tumours have therefore a greater possibility of setting up secondaries in the liver than those drained by the vena cava Cells from other tumours except from primary pulmonary carcinoma must pass the lungs before they reach the liver The material was therefore divided into 3 groups according to *Walther* (1948)

1 Tumours drained by the vena portae
2 Pulmonary tumours
3 Tumours drained by the vena cava

The distribution among these 3 groups is given in Table 4 from which it is clear that the subjects with cirrhosis did not differ significantly from the entire material regarding the sites of the tumours

TABLE 4
Sites of Extra Hepatic Carcinomas

	Drained by vena portae	Pulmonary carcinoma	Drained by vena cava
All	40 0 %	14 3 %	45 7 %
With cirrhosis	43 9 %	14 0 %	42 1 %

TABLE 5
Metastases from Different Primary Tumours

	Cirrhosis	Not cirrhosis	Cirrhosis	Not cirrhosis
	Liver metastases		*Pulmonary metastases*	
Drained by vena portae	56 0 %	60 8 %	48 0 %	29 [illegible] %
	60 6 %		30 0 %	
			Adrenal metastases	
Pulmonary carcinoma	25 0 %	49 3 %	25 0 %	36 7 %
	48 7 %		36 4 %	
			Pulmonary metastases	
Drained by vena cava	29 [illegible] %	41 3 %	37 5 %	58 1 %
	44 0		57 6 %	

Mean Number of Sites of Metastases

	Drained by vena portae	Pulmonary carcinoma	Drained by vena cava
Cirrhosis	2 6	2 6	3 0
Not cirrhosis	3 [illegible]	4 0	4 1
Total (cirrhosis + not cirrhosis)			

This means that there was no evidence of liver cirrhosis favouring development of carcinomas in such organs as were drained by the vena portae

Table 5 shows that pulmonary carcinoma as well as tumours drained by the vena cava set up secondaries less often in the cirrhotic than in the non-cirrhotic liver A corresponding difference was however also found in the frequency of extrahepatic metastases This difference is also seen in the frequency of metastasis in arbitrarily selected organs such as the adrenals in pulmonary cancer and the lungs in tumours drained by the vena cava These differences can be explained entirely by the fact that the noncirrhotic cases represented a more advanced stage of metastasis than the group with cirrhosis However in subjects whose tumours were drained by the vena portae pulmonary metastases were more common in the presence of cirrhosis This difference ($0.05 > p > 0.02$) is remarkable It is the opposite of what should have been expected since the tumours in the cirrhosis group were in an earlier stage of metastasis

If the cirrhotic liver allows the passage of tumour cells more readily than the normal liver the cirrhotic liver parenchyma may not be reached by so many cells from tumours drained by the vena portae Despite this and despite the fact that the tumours in the cirrhosis group were in an earlier stage of metastasis the frequency of liver metastases was the same in both groups

DISCUSSION

The results of the experimental investigation suggest that tumour cells injected into the vena portae produce tumours equally often in portal as in central liver cirrhosis The fact that human liver cirrhosis is as a rule portal while liver cirrhosis in experimental animals hitherto examined was of central type can thus hardly explain the described difference between the incidence of metastasis in experimental and human liver cirrhosis When the tumour cells were injected directly into the liver no difference between animals with and without cirrhosis was found This suggests that the higher frequency of metastasis in the liver with cirrhosis when tumour cells are injected intraportally might be due to a vascular factor

Earlier investigations suggest that it is not a question of a decreased passage of tumour cells into the cirrhotic liver but instead a change in the distribution of the tumour cells in the liver (*Saldeen* 1963) In unpublished experiments the number of intraportally injected tumour cells (living or fixed and stained) found in the liver sinusoids was smaller in rats with than in those without cirrhosis

According to some earlier autopsy series (Table 6) secondaries are rarely seen in cirrhotic livers (*Leber* 1957 *Chomet et al* 1959 *Gall* 1960 *Fisher et al* 1960 *Ruebner et al* 1961) while others show no

difference in this respect between the normal and the cirrhotic liver (Norkin et al 1962 Goldstein et al 1966) *Fisher et al* (1960) and *Ruebner et al* (1961) however pointed out that tumour cases with cirrhosis more often have no metastases and this might explain the difference found

TABLE 6

Frequency of Liver Metastases in Subjects with and without Cirrhosis

		Cirrhosis	Not cirrhosis
Lisa et al	(1942)	27.2 %	33.0 %
Lieber	(1957)	6.7 %	28.6 % (total)
Call	(1960)	10.1 %	46.3 %
Ruebner et al	(1961)	20.4 %	36.1 %
Norkin et al	(1962)	35.7 %	35.3 %
Fisher et al	(1960)	64.7 %	76.8 %
Present series		21.3 % (40.4 %)	35.5 % (51.6 %)

Frequency among metastasising tumours

The results presented in previous investigations are however often difficult to evaluate because *inter alia* of the smallness of the groups and the heterogeneity of the material (different hospitals with different frequencies of cirrhosis and cancer) Moreover in such comparisons the authors have often failed to take into account differences as regards the sites of the individual tumours the number of metastasizing tumours and their stage of metastasis

Ruebner et al (1961) pointed out that the *frequency of metastasis* of extrahepatic cancer is low in patients with liver cirrhosis but when a tumour has once metastasized the chances of an establishment of secondaries in the liver were found to be equal in patients with and without cirrhosis Their control series consisted of sex and age matched subjects without liver cirrhosis The frequency of liver metastasis in the control series however differed from that in the entire material In our opinion it is better to use a large control series which provides the possibility of comparing tumours in the same sites and in the same stage of metastasis Our results reveal that the previously reported tendency of metastasis to be less common in cirrhotic livers is due to the use of unsuitable methods in the statistical analysis of the data The difference found can very well be explained by the fact that many tumours in patients with liver cirrhosis have not metastasized and several are in an earlier stage of metastasis at death and thus that the cirrhotic liver is not reached by circulating tumour cells to the same extent As to tumours drained by the vena portae local circulatory conditions (intrahepatic Eck fistulae) may also explain why the liver parenchyma is not reached by these tumour cells This may also be the reason why tumours drained by the vena portae more often metastasize to the lungs in the presence of cirrhosis The observed significant difference in the frequency of hepatic metastasis in subjects with and

without cirrhosis was no longer demonstrable when the differences in the total frequency of metastasis in the two groups were taken into account in the statistical analysis Thus by comparison of tumours with hepatic respectively extrahepatic metastases only carcinomas in early stage of metastasis were more often found in subjects with cirrhosis than in those without In subjects without cirrhosis 12 per cent of the carcinomas in advanced stage of metastasis had not spread to the liver while in the cirrhosis group all tumours in advanced stage of metastasis had given hepatic metastases The investigations suggest that if tumour cells once reach the liver parenchyma they set up secondaries more often in the cirrhotic than in the non-cirrhotic liver

TABLE 7

Frequency of Liver Cirrhosis and Extra Hepatic Cancer

		Cases with cirrhosis and extra hepatic carcinoma	Cirrhosis	Carcinoma total	Extra hepatic carcinoma	
					Cirrhosis	Not cirrhosis
Lieber	(1957)	89	3 0 %	17 1 %	8 6 %	17 1 %
Call	(1960)	109	–	–	15 8 %	[illegible] 0 %
Fisher et al	(1960)	28	7 4 %	39 0 %	13 1 %	33 5 %
Ruebner et al	(1961)	54	1 7 %	–	13 5 %	27 0 %
Vorkin et al	([illegible])	121	6 7 %	18 1 %	9 5 %	18 5 %
Present series		108	3 8 %	34 2 %	24 2 %	33 9 %

37 2 per cent when stillborns and newborns were not included

TABLE 8

Mean Age of Subjects with Cirrhosis and/or Carcinoma

		Cirrhosis	Cirrhosis + carcinoma	Carcinoma
Fisher et al	(1960)	52 6 years	61 7 year	57 7 years
Ruebner et al	(1961)	5? 3 years	?1 9 years	5? 7 years
Vorkin et al	(196?)	app ?? years	65 ? years	app 6 years
Present series		68 ? years	71 6 years	70 ? years

Extra hepatic carcinoma

A noteworthy observation in the present material was the lower frequency of extrahepatic cancer in patients with liver cirrhosis (24 2 per cent) than in patients with a normal liver (37 2 per cent when stillborn and newborn were not included) This lower frequency has also been observed by previous workers in this field (Table 7)

This difference cannot be ascribed to differences in age Both in our material and in previous series (Table 8) subjects with combined liver cirrhosis and cancer appeared if anything to be older than patients with cancer only

Among subjects at least 40 years of age and without cirrhosis extrahepatic carcinoma was found in 3 196 (No subject below 40 years had liver cirrhosis) During the 8 years (1958–1965) altogether 15 250 subjects in this age group died in Malmo At least 385 of these had cirrhosis but even if it be assumed that all the subjects with extrahepatic cancer as well as all subjects with cirrhosis had been included in the autopsied group the total frequency of extrahepatic cancer in subjects without cirrhosis would have been 21 3 per cent (3 196 out of 14 865)

The true frequency must of course have been higher There is no reason for instance why microscopic cancer of the prostate should be less common among the 3 446 males in this age group not autopsied (if anything it was probably more common since most of the men not autopsied belonged in the highest age group (>80 years)) On this assumption the frequency of extrahepatic carcinoma would rise from 21 3 per cent to 24 7 per cent Further carcinomas would have been revealed if a systematic autopsy had been performed and we therefore think that the difference in frequency of extrahepatic carcinoma in subjects with respectively without cirrhosis is a true one It is however impossible exactly to assess the difference

It thus appears as if extrahepatic cancer develops less often in patients with cirrhosis It is for example known that patients with cirrhosis have a high serum oestrogen concentration The decreased frequency of extrahepatic cancer however appears to apply to all carcinomas We have not been able to demonstrate any difference between hormone dependent carcinoma such as prostatic cancer and mammary cancer and other types of carcinoma

It is also known that the fibrinolytic activity in the blood is increased in liver cirrhosis (*e g Kwaan et al* 1956) and it has been shown experimentally that tumour cells take less often in animals treated with fibrinolysin than in untreated ones (*e g Clifton & Agostino* 1962 1963)

The lower frequency of carcinomas found in patients with cirrhosis is also of interest in the discussion of metastasis It cannot be excluded that the same mechanism is responsible for the suppression of the frequency of tumour as well as of the metastases in those patients in whom tumour nevertheless developed

Patients with carcinoma and coexisting cirrhosis have tumours which at the time of death of the patients are in an earlier stage of metastasis than are the tumours in patients without cirrhosis This might be explained by the fact that the duration of survival may be shorter in patients with coexisting liver cirrhosis and cancer than in those with cancer only *Ruebner et al* (1961) found for example that patients with cirrhosis survived for 11 months after the operation for cancer (50 per cent died within 3 weeks) while those without cirrhosis survived for 16 months (50 per cent died within 3 months)

SUMMARY

Previous experimental investigations have shown that tumour cells produce tumours more often in the cirrhotic liver than in the normal Statistical analyses of human autopsy series have however usually shown that metastases are more common in non cirrhotic livers

This disagreement can probably not be explained by the fact that human cirrhosis is usually of the portal type while in most of the experiments on record it was of the central type because judging from the present investigation injections of tumour cells set up tumours equally often in livers with experimental portal cirrhosis as in those with central cirrhosis

Previous statistical analyses of findings at autopsy of human material were often carried out without due consideration to site stage of metastasis and general tendency of the tumours to metastasize When these factors were taken into account in the present autopsy series it was found that metastasis from extrahepatic carcinoma to the liver is not less common in the presence of cirrhosis The difference found suggests that once tumour cells reach the liver parenchyma they set up growths more often if the liver is cirrhotic than if it is not

REFERENCES

Ahlström C C & Jonsson N Induction of sarcoma in rats by a variant of Rous virus Acta path microbiol scandinav 54 145–172 1962

Campbell J A H Experimental non dietary cirrhosis in rats Brit J exp Path 42 290 295 1961

Chomet B Valaitis J & Pearah C Metastatic carcinoma in the cirrhotic liver Am J med Sci 238 753–759 1959

Clifton E E & Agostino D Factors affecting the development of metastatic cancer Effect of alterations in clotting mechanism Cancer 15 276–283 1962

Clifton E E & Agostino D Irradiation and anticoagulant therapy to prevent pulmonary metastases of the V 2 carcinoma in rabbits Radiology 80 236 243 1963

Fisher E R & Fisher B Experimental studies of factors influencing hepatic metastases IV Effect of cirrhosis Cancer 13 860–864 1960

Fisher E R Hellstrom H R & Fisher B Rarity of hepatic metastases in cirrhosis A misconception JAMA 174 366–369 1960

Gall E A Primary and metastatic carcinoma of the liver Relationship to hepatic cirrhosis Arch Path 70 226–232 1960

Goldstein M J Frable W J & Sherlock P Hepatic metastases and portal cirrhosis Am J med Sci 252 26–30 1966

Kwaan H C McFadzean A J S & Cook J Plasma fibrinolytic activity in cirrhosis of the liver Lancet 1 132 1956

Lieber M M The rare occurrence of metastatic carcinoma in the cirrhotic liver Am J med Sci 233 145–152 1957

Lisa J R Solomon C & Gordon F J Secondary carcinoma in cirrhosis of the liver Am J Path 18 137–140 1942

Madden R E & Burk D Production of viable single cell suspensions from solid tumours J Nat Cancer Inst 27 841–861 1961

Norkin S A Heimann R & Falcini H D Neoplasms cirrhosis and hepatic metastasis Cancer 15 1004–1008 1962

Ruebner B H Green R Miyai K Caranasos G & Abbey H The rarity of intrahepatic metastasis in cirrhosis of the liver A statistical explanation with some comments on the interpretation of necropsy data Am J Path 39 739–746 1961

Saldeen T Experimental studies on spread of Rous sarcoma in rats Acta path microbiol scandinav Suppl 167 1 87 1963

Schrek R A quantitative study of the growth of the Walker rat tumour and the Flexner Jobling rat carcinoma Amer J Cancer 24 807–822 1935

Schrek R A method for counting the viable cells in normal and malignant cell suspensions Amer J Cancer 28 389 392 1936

Walther H E Krebsmetastasen Benno Schwabe & Co Basel 1948 p 106

Acta path microbiol scandinav 74 353-361 1968

Department of Pathology Sentralsykehuset i Trondheim Norway
(Head Erling A Mylius MD)

ULTRASTRUCTURAL FEATURES OF CHANG HUMAN LIVER CELLS IN MEDIA OF LOW GLUCOSE CONTENT

A Comparison with HeLa Cells Cultivated under Similar Conditions

By

MARGRETE WILLOCH[1]

Received 1 ix 68

HeLa cells have been transferred from a standard nutritive medium containing 100 mg per cent glucose to an experiment medium containing 6 mg per cent glucose Following transfer the cells were subjected to an electron microscope study to reveal possible ultrastructural changes indicative of a metabolic shift connected with a limitation of accessible glucose (Willoch 1967 a b) The ultrastructural response of HeLa cells to a reduced glucose supply proved to comprise two separate sets of reactions

1) A transitory change of about 1 week s duration comprising increased density of the mitochondrial matrix dilation of the dictyosomal cisternae a more organized pattern and increased granulation of the ER membranes and an increase in number of lysosome like bodies accompanied by a transition in their appearance from multi vesicular bodies to inclusions containing membrane elements and highly dense areas
2) A permanent overall reduction in number and extension of the cytoplasmic protrusions a more regular and uncomplicated cell surface and smooth intercellular borders equipped with desmosomes

The former modifications were assumed to represent a shock or relaxation reaction directly connected with the shift in environmental conditions The latter reaction was interpreted as an expression of a reduced transport of metabolites across the cell membrane reflected by a reduction of the cell surface area

The present report deals with the ultrastructural features of Chang human liver cells in short and long term cultures in both 100 mg per

[1] Fellow of the Norwegian Cancer Society

cent glucose and 6 mg per cent glucose compared with the reported observations on HeLa cells For comparison some of the organelle modifications reported for HeLa cells are included in the illustrations of this paper

MATERIAL AND METHODS

Techniques for preparation of media containing 100 mg per cent and 6–7 mg per cent glucose cultivation of HeLa cells fixation preparation of thin sections and surface replica specimens and electron microscopy have been described in an earlier paper (Wallach 1967 b)

Chang human liver cells were treated according to the same procedures except that versene disaggregation experiments were not carried out

The reported results are based on the observation of 6 chronologically separate samples of cells fixed 3 days after transfer from standard medium to experiment medium 6 samples of cells cultivated in experiment medium for more than 3 weeks before fixation and 6 (Chang) or 15 (HeLa) samples of cells cultivated continuously in standard medium Each sample comprised pooled fixed cell masses from 5 roller tubes.

RESULTS

The experimental period lasted for 4 months during which cells cultivated in experiment medium maintained an unchanged growth rate identical with that of cells cultivated under standard conditions No change in growth pattern or internal structure of the cells could be detected by light microscopy of living cultures or of fixed and stained specimens

Ultrastructurally the following description applies to Chang cells under all environmental conditions created in the present study and at all observed stages of experiment

Mitochondria are elongate irregularly twisted measuring 0 20–0 35 microns in width (Figs 1 and 2) The cristae have no specific orientation with respect to the mitochondrial axis though longitudinal cristae seem to be predominant The mitochondrial matrix is frequently though not invariably dense and irregularly granulated This density tends to obscure the outline of the cristae

---

Figs 1–4

Fig 1 Mitochondrion in Chang cell standard medium The mitochondrial matrix is somewhat denser than the surrounding cytoplasm partly obscuring the outline of the transversal cristae × 75 000

Fig 2 Mitochondria in Chang cell experiment medium Cristae appear to be transversal oblique and longitudinal The mitochondria are smaller than that shown in Fig 1 This difference is not related to difference in nutritive medium. × 75 000

Fig 3 Mitochondrion in HeLa cell standard medium The matrix appears to be of the same density as the surrounding cytoplasm. No specific orientation of the cristae is noted. × 75 000

Fig 4 Mitochondria in HeLa cell cultivated for 3 days in experiment medium The matrix is very dense and outline of cristae is not distinct. × 75 000

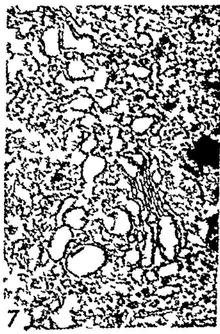

1


3




4


6


7

The Golgi substance is extensive but appears to be located in a fairly well circumscribed area It consists of numerous small vesicles and several stacks of flat cisternae which show occasional dilations The cytoplasm of this area is vacuolated to a higher degree than the remaining cytoplasm (Fig 5)

The cells contain a fairly small number of heterogeneously dense lysosome like bodies which contain membrane elements vesicles granula and irregular dense masses Typical multi vesicular bodies have not been observed

The endoplasmic reticulum is unconspicuous consisting of irregularly scattered apparently short membranes which are relatively sparsely granulated (Figs 8 and 10)

There is a marked general granulation of the cytoplasm

Microvillous protrusions extend from all parts of the free cell surface including the rim of the cells The protrusions contain no organelles or other specific structures At their basis structures indicative of pinocytotic activity are occasionally observed (Fig 8) Measured in thin sections the protrusions have a fairly constant diameter of 0 05–0 1 microns (Fig 8) In surface replicas protrusions of the upper free surface are 0 15–0 20 microns in width (Fig 11) whereas the cell periphery is seen to extend into flattened branching protrusions of highly variable width indicating that the peripheral protrusions may participate in the attachment or locomotion of the cells (Fig 12)

The intercellular space consists of protrusion filled gaps alternating with areas of close intercellular contact along straight smooth borders which rarely exceed 2–3 microns in length (Figs 9 and 10)

DISCUSSION

Available literature contains no information on the glucose metabolism of Chang liver cells cultivated in media with a glucose concentration comparable to the experimental concentration applied in the present study

There is little reason to assume that the functional significance of the cytoplasmic protrusions of the upper free cell surface is essentially different in Chang and HeLa cells The protrusions are presumably of

---

Figs 5–7

Fig 5 Golgi zone in Chang cell experiment medium The cytoplasm of this area is vacuolated Some of the Golgi cisternae show dilations Golgi zones in Chang cells cultivated in standard medium are of the same appearance × 40 000

Fig 6 Part of Golgi zone in HeLa cell standard medium The cytoplasm is vacuolated cisternae are flat × 45 000

Fig 7 Part of Golgi zone in HeLa cell experiment medium In addition to vacuolization of the cytoplasm there are numerous dilations of the cisternae × 45 000

functional importance in the transport of metabolites across the cell membrane (*Fisher & Cooper* 1967) and there is probably a parallelism between the amount of metabolites transported and the quantitative development of protrusions (*i e* surface area of the cells) (*Willoch* 1967 b) There is evidence that the glucose metabolism of Chang cells responds to environmental changes in a way different from other cells *in vitro* (*Pihl & Eker* 1963) The present observation of a lack of change in surface ultrastructure of Chang cells at transition to low glucose concentration may suggest that Chang cell glucose metabolism is quantitatively less sensitive to great variations in the amount of accessible glucose than that of HeLa cells

The ultrastructure of all internal organelles of Chang cells is observed to be unaffected by the reduction of glucose supply both immediately after transition and during prolonged growth in experiment medium The shift in environmental conditions does not seem to entail a shock or metabolic relaxation similar to that ascribed to HeLa cells subjected to the same treatment

While HeLa cells are of malignant origin Chang cells derive from normal human liver The present comparison can not be directly correlated with the malignant/normal origin of these two cell types as there seems to be no regular difference in metabolic reaction pattern between malignant and normal cells *in vitro* when their glucose supply is varied (*Danes & Paul* 1961 *Eagle et al* 1958) The comparison remains confined to HeLa and Chang cells as such and the observations may imply the suggestion that HeLa cells are metabolically more dependent than Chang cells on the amount of accessible glucose

There exists an unsatisfied demand for simple unambiguous morphological criteria for the identification of origin and type of cells *in vitro* (*Harris* 1964 *Willmer* 1965) According to the present observations Chang and HeLa cells are ultrastructurally grossly similar under standard conditions and can not be distinguished from each other by means of simple electron microscopy Also the variability of HeLa cell ultrastructure under varying but sufficient nutritive conditions leaves little possibility for the establishment of specific ultrastructural characteristics of this cell

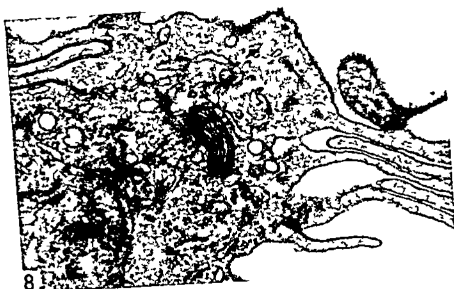
---

Figs 8–10

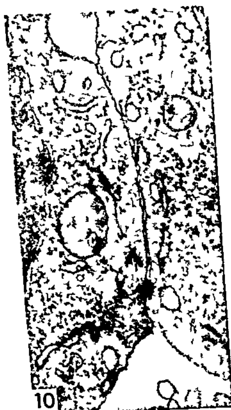
Fig 8 Part of periphery of Chang cell standard medium The long slender cytoplasmic protrusions have a uniform width of 0 05 0 1 micron Structures indicate of pinocytotic activity are seen at the basis of some protrusions Note elements of the endoplasmic reticulum × 45 000

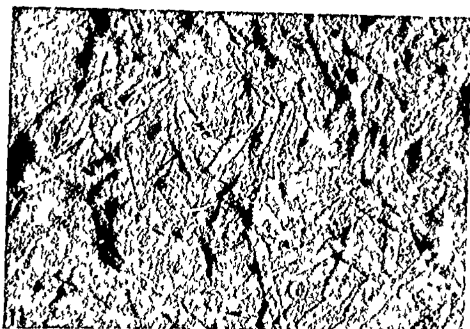
Fig 9 Straight uncomplicated part of intercellular space 2 2 microns in extension Chang cells standard medium × 40 000

Fig 10 Straight uncomplicated part of intercellular space 1 6 microns in extension Chang cells experiment medium The cytoplasm contains small elements of the endoplasmic reticulum × 45 000


8


9


10





Figs 11–12

Fig 11 Part of upper free surface of Chang cell The collapsed microvillous protrusion have a fairly constant width of 0 15–0 20 microns × 18 500

Fig 12 Peripheral protrusion of Chang cell Peripheral protrusions are pseudopodia like branched and of highly variable width indicating that they may be of importance in the attachment and locomotion of the cells × 15 000

SUMMARY

An electron microscope study has been made of external and internal morphological features of Chang human liver cells cultivated in a standard nutritive medium containing 100 mg per cent glucose and in an experiment medium containing 6 mg per cent glucose The observa

tions have been compared with those reported earlier for HeLa cells subjected to the same treatment

Chang cell surfaces exhibit numerous filiform cytoplasmic protrusions which are apparently modified into flat pseudopodia like protrusions along the cell rim Internal organelles are of little specific appearance and show no sign of orientation or specific location

In HeLa cells a reduction of glucose supply induces ultrastructural changes suggestive of an immediate shock reaction and a permanent reduction of surface area induced by a decrease in metabolic activity In contrast all ultrastructural features of Chang cells remained unchanged during the entire experiment period It is suggested that Chang cells are less sensitive than HeLa cells to great variations in environmental glucose concentration possibly in the sense of being less dependent on the amount of accessible glucose in the quantitative maintenance of their glucose metabolism

It is also indicated that neither of the two cell types show ultrastructural characteristics suitable as specific identification markers

REFERENCES

1 *Danes B S & Paul J* Respiration of normal and tumor cells in culture Path Biol *9* 571–573 1961

2 *Eagle H Barban S Levy M & Schulze H O* The utilization of carbohydrates by human cell cultures J Biol Chem *233* 551–558 1958

3 *Fisher H W & Cooper T W* Electron microscope studies of the microvilli of HeLa cells J Cell Biol *34* 569–576 1967

4 *Harris M* Cell culture and somatic variation pp 178–182 New York 1964

5 *Pihl A & Eker P* Studies on the action of adrenal steroids on mammalian cells in tissue culture S and J Clin Lab Invest *15* Suppl 76 67–68 1963

6 *Willmer E N* Morphological problems of cell type shape and identification In Cells and tissues in culture E N Willmer ed pp 141–176 London 1965

7 *Willoch M* Transient modification of the HeLa cell organelles following reduction of glucose supply Acta path microbiol scandinav *69* 618–619 1967 a

8 *Willoch M* Changes in HeLa cell ultrastructure under conditions of reduced glucose supply Acta path microbiol scandinav *71* 35–45 1967 b

Acta path. microbiol. scandinav. 74: 36?–370, 1969

The Department of Bacteriology, Statens Bakteriologiska Laboratorium,
Stockholm 1, Sweden

IMPROVED METHODS FOR GONOCOCCAL SAMPLING AND EXAMINATION ON A LARGE SCALE

By

B. Gästrin and L. O. Kallings

Received 27. ii. 68

Due to the high frequency of gonorrhea in Sweden and to the corresponding activities to treat and control the disease (*Gästrin & Kallings* 1964), the laboratory methods were analysed in an effort to find more sensitive procedures and adapt them to large scale operations. Although the bacteriological methods for examining gonococcal samples have steadily improved during recent years, many cases of gonorrhea still escape diagnosis because of the difficulty in keeping the gonococci alive during transport to the laboratory and obtaining recognizable growth on the culture media. Since 1946 *Stuart* has studied this transport problem and in a series of articles he reported the experience with a medium which now is used in many laboratories throughout the world (*Stuart* 1946, 1956, 1959). The results have been confirmed by *Reyn* (1960) and *Ringertz* (1960) among others. For a laboratory to serve large districts it is necessary to improve the transport medium to meet the demands of more protracted carriage and permit long storage periods of the medium so that physicians will have a transport kit always at hand. Under these conditions one can rationalize the large scale production and distribution of this medium.

A number of selective cultivating media had been tested earlier with a view to their capacity of inhibiting irrelevant bacteria in the specimens without seriously inhibiting the gonococci, but none proved satisfactory (*Crookes & Stuart* 1959). In 1964, however, *Thayer & Martin* reported favourable results with a medium containing polymyxin and ristocetin (*Thayer & Martin* 1964, 1966, *Martin et al.* 1965). Later ristocetin was exchanged for vancomycin (*Martin et al.* 1967).

Although the optimal environmental factors for cultivation of gonococci are well known, practical difficulties of large scale cultivation are involved in the maintenance of optimal cultivation conditions over the entire incubation period.

The present paper describes the results with a new transport kit (*Kallings* 1968) designed for the sampling and forwarding of gono-

coccal specimens Different materials and sterilization methods for the swabs also were tried Further the selective medium containing poly myxin and ristocetin was studied on a large number of samples The maintenance and control of suitable environmental conditions for the cultivation of a large number of gonococcal samples (500–700 samples/day) was solved by using an incubator with a controlled level of CO and humidity in the atmosphere

MATERIALS AND METHODS

Gonococcal strains The investigations were performed on gonococcal strains isolated *from routine tests on samples sent to the laboratory* These strains were not subcultured more than 5 times during the experiments The verification criteria for diagnosis were typical colony formation with oxidase positive gram negative diplococci which *ferment dextrose but not levulose* or maltose

Transport medium Stuart s medium modified by *Ringert* (1960) was used as transport medium and was made according to the following procedure Agar 10 g thioglycollic acid 1 ml Na *glycerophosphate (20 per cent in water)* 50 ml CaCl solution (1 per cent in water) 10 ml methylene blue solution (0 1 per cent in water) *2 ml* aq dest 950 ml

Cultivation medium The cultivation medium was *haematin agar with horse* serum instead of ascitic fluid according to the following formula Spanish agar 6 8 *g ox heart broth 1000* ml peptone (Wilson) 10 g NaCl 3 g Na HPO_4 12 H O 2 g whole horseblood (heated to 78 C) 85 ml horse serum 100 ml The pH of the medium was 7 2 The selective medium consisted of the same haematin agar with 25 units *polymyxin and 10* mcg *ristocetin/ml*

Diluent Phosphate buffered saline (PBS) according to the following formula NaCl 8 g Na HPO_4 2 H O 1 1 g KCl 0 2 g KH PO 0 2 g aq dest 1000 ml

Transport kit In the new *kit 20 ml of the transport* medium was filled in a glass ampoule of about 150 mm length and 15 mm diameter (*Kallings* 1958) The air in the ampoule was substituted by nitrogen gas the ampoule was sealed and autoclaved The swab was wrapped in a *plastic package and enclosed* with the ampoule in a special polystyrene transport tube (Fig 1)

Swabs The *routine* procedure used earlier involved boiling ordinary cotton tipped wooden swabs in Sørensen phosphate buffer *for 5 minutes followed by dipping* in a suspension of 2 per cent activated charcoal in distilled water and autoclaving at 120 *C for 20 minutes* Later the specimen swabs for routine use were sterilized by gamma irradiation Five different types of plastic polymers *sterilized by gamma* irradiation have been tested as material for the transport swab sticks

Environmental factors The *earlier routine method* involved incubation of the plates for 1 day in a closed container of stainless steel (55 × 30 × 25 cm) with a capacity *of 150 plates* CO_2 was produced by mixing an excess of sodium bicarbonate and 50 ml of sulphuric acid in a dish on the bottom of the container the humidity was raised by means of a sponge placed in a vessel containing water Then the plates were incubated *for* another day in an ordinary incubator The incubation temperature was 37 C but it took a considerable time to reach this temperature throughout the container

In order to obtain better control *of temperature* CO and humidity a CO_2 incubator with a capacity of 2 000 agar plates is being used now (Carbon Dioxid Incubator No *1236* manufactured by Hotpack Corp U S A) The humidity is regulated automatically The CO content is regulated by *the inflow of air* and CO *in a fixed* proportion and can be varied between 0 and 20 per cent CO_2 As a result of experimentation a *relative humidity* of 90–95 per cent and a CO content of 4–10 per cent was used For the routine isolation of gonococci the plates were incubated for about 36 hours at 36 5 ± 0 5 C

The temperature was measured with *thermocouples containing* 10 sensitive measuring elements The CO content in the atmosphere was determined by a Drager measuring apparatus (*Dragerwerk Lübeck*) with a reagent tube The relative humidity was determined with an electric hygrometer

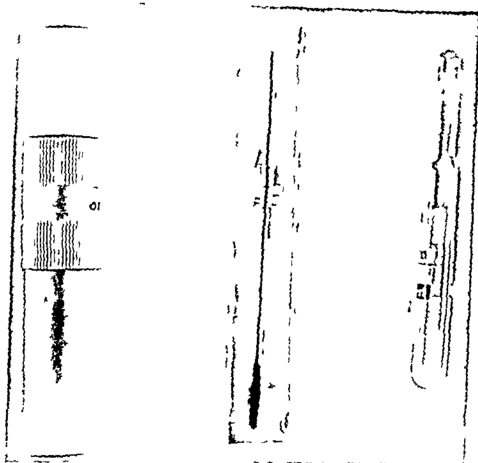


Fig 1

Transport kit for gonococcal specimens From left to right Transport container (mailing tube) sterile specimen swab in plastic envelope and medium ampoule

Bacterial counting The survival of the gonococci in the transport medium was investigated in the following manner The gonococci which had been grown on haematin agar plates for 18 hours were suspended in PBS to a certain density 0 1 ml aliquots of this suspension were absorbed by carbon coated swabs which then were inserted into the transport medium and allowed to remain there for 1 24 and 48 hours at 22 C. The swabs were removed from the transport medium and shaken mechanically in 5 ml PBS under standardized conditions after which the number of bacteria in this solution was counted by means of the plate dilution method Aliquots of 0 2 ml of ten fold step dilutions were distributed over the surface of haematin plates The colonies on plates giving between 30–300 colonies were counted after incubation for 20 hours at 37 C in CO

In the experiments concerning various materials and sterilization methods of swabs the swabs were tested by the above procedure The storage time in the transport medium was always 48 hours and in all experiments wooden swabs sterilized by heating were used as controls

RESULTS

The Transport Medium

After various periods of storage at room temperature the medium sealed in ampoules was compared with fresh medium. The average of duplicated experiments with ten newly isolated gonococcal strains were used for each of the comparisons. The results are given in Table 1 as the quotient of the number of surviving bacteria in the ampoule medium to that in the fresh medium. The ampoule medium was found to be better than fresh medium even if the ampoules were stored for 42 months at about 22° C.

TABLE 1

Comparison between Fresh Stuart Medium and Stuart Medium Stored in Sealed Ampoules for Different Periods of Time

Storage time of medium in months	Storage time of bacteria in medium ("transport time")			Average
	1 hour	24 hours	48 hours	
3	1 0	3 3	1 [illegible]	2 0
5	0 7	1 [illegible]	1 3	1 1
9	[illegible] 2	1 4		1 8
12	1 1	3 5	3 3	2 [illegible]
20	1 1	1 4	1 [illegible]	1 3
42	1 7	1 9	2 1	1 9

Average of all quotients 1 8

The values represent the quotient $\frac{\text{bacterial no in ampoule medium}}{\text{bacterial no in fresh medium}}$

The Swabs

Five different types of plastic polymers were tested as material for the sticks and were compared with wooden ones. The polymers were considered to be nontoxic. The survival on plastic swabs of 10 newly isolated gonococcal strains was investigated in duplicate after storage in Stuart's medium at 22° C for 48 hours. In each test of the plastic swabs wooden swabs were included as controls. The results are given as the average of the number of bacteria/ml of solution in which the sticks were shaken to release the bacteria (Table 2). The results show that the ordinary wooden sticks were considerably better than the plastic sticks tested.

When specimen swabs sterilized by gamma radiation were used bacterial survival was tested in the same manner as bacterial survival on the plastic sticks. Gonococcal viability on wooden swabs whether treated with a radiation dose of 4 5 megarad or heat sterilized was not found to differ.

TABLE 2

Tests with Different Plastic Stick Swabs

Type of plastic material	No of bacteria/ml in shaking solution	
	Plastic swabs	Wooden swabs
Polypropylene I	390	3 100
Polypropylene II	610	5 100
Low pressure polyethylene	3[illegible]0	5 100
Nylon I	270	40 000
Nylon II	410	40 000

The values given represent the mean number of bacteria/ml in the shaking solution in experiments with 10 gonococcal strains after 48 hours storage in Stuart medium

The Selective Medium

The comparison of the ordinary haematin medium with the selective medium containing 25 units of polymyxin and 10 mcg ristocetin per ml was first carried out on about 8 500 routine samples received during some weeks The results encouraged further studies during an additional limited period of time on about 11 000 samples These comparisons showed that about 20 per cent of the gonococcal findings could be demonstrated by the help of the selective medium only About 14 per cent were demonstrated exclusively on the ordinary haematin agar For epidemiological reasons with a view to revealing as many gonorrheal cases as possible it was decided to use both ordinary and selective medium in the routine over a longer period Thereby a total of 94 379 samples were made available for analysis Gonococci were demonstrated in 9 567 or 10 1 per cent of the samples in 6 641 of these (7 1 per cent of all samples) gonococci grew both on the ordinary haematin agar and on the selective medium in 818 samples (0 9 per cent of all samples) gonococci grew only on haematin agar and in 2 108 samples (2 2 per cent of all samples) gonococci grew only on the selective medium (Table 3)

TABLE 3

Comparison of the Ability to Isolate Gonococci from Samples on Ordinary Haematin Agar Plates and Plates Containing the same Medium with the Addition of 25 Units Polymyxin and 10 µg Ristocetin per ml (Selective Medium)

	Number of *samples*	Per cent of samples *positive*
Total samples	94 379	
Total positive samples	9 567	10 1
Growth of gonococci on *both media*	6 641	7 1
Growth of gonococci on haematin agar only	818	0 9
Growth of gonococci on selective medium only	2 108	2 2

To check that the increased number was not due merely to the fact that two plates instead of one were used to test the samples a comparison was done with 11 000 specimens cultured on two plates containing the same medium The comparison showed that the number of positive samples were increased by 0.2–0.4 per cent if duplicate plates of the same medium were used

Growth of contaminating bacteria on the two media differed greatly The ordinary medium was reported to be overgrown in 9.8 per cent of the samples but only in 0.9 per cent on the selective medium (calculated from 10 400 samples) The selective medium inhibited growth of almost all contaminating bacteria with the exception of some strains of proteus In many instances, the gonococci were found in pure culture on the selective medium

TABLE 4

Comparison of the Ability to Isolate Gonococci after Incubation in a Conventional Box (see Text for Description) and in a CO_2 Incubator Maintaining Optimal Environmental Conditions

	Total no of samples	No of positive samples	Per cent positive samples
Incubation in box	55 [illegible]4	5 226	9.4
Incubation in CO_2 incubator	38 815	4 341	11.2

The selective medium only was incubated in the CO_2 incubator whereas the ordinary haematin agar was still incubated in boxes

Environmental Factors

An investigation of the effect of environmental factors on the growth of gonococci showed that an effective regulation of the optimal conditions as regards temperature relative humidity and CO_2 content increased the number of positive findings by 1.8 per cent (Table 4) compared with that obtained by the conventional culture method The colony diameter of 10 newly isolated strains was measured after 20 hours incubation such organisms as had been cultivated under controlled conditions had diameters twice the size of those of organisms incubated in steel boxes placed in an ordinary incubator in which a humid CO_2 rich atmosphere was produced by simple methods An investigation of the temperature in the medium within the petri dishes in the box and in the CO_2 incubator showed that a temperature of 36 C was reached in the latter about 1 hour after it was loaded with 2 000 agar plates at room temperature However heating in the steel box loaded with 150 plates proceeded at a considerably lower rate

10 *Ringert O* A Modified Stuart Medium for the Transport of Gonococcal Specimens Acta path microbiol scandinav 49 105–112 1960
11 *Rubbo S D & Benjamin M* Some Observations on Survival of Pathogenic Bacteria on Cotton Wool Swabs Brit Med J 1 983–987 1951
12 *Stuart R D* The Diagnosis and Control of Gonorrhoea by Bacteriological Cultures Glasgow Med J 27 131–142 1946
13 *Stuart R D* Transport Problems in Public Health Bacteriology Canad J Publ Health 47 114–122 1956
14 *Stuart R D* Transport Medium for Specimens in Public Health Bacteriology Publ Health Rep 74 431–438 1959
15 *Thayer J D & Martin J E Jr* A Selective Medium for the Cultivation of N *gonorrhoeae* and N *meningitidis* Publ Health Rep 79 49–57 1964
16 *Thayer J D & Martin J E Jr* Improved Medium Selective for Cultivation of N *gonorrhoeae* and N *meningitidis* Publ Health Rep 81 559–562 1966
17 *White W D* Antibacterial Bacteriological Swab Brit Med J 2 229–230 1965

ADDENDUM

In October 1968 ristocetin is still available and continuation of production is planned

Acta path microbiol scandinav 74 371-380 1968

Statens Bakteriologiska Laboratorium Stockholm Sweden

THE SURVIVAL TIME FOR DIFFERENT BACTERIA IN VARIOUS TRANSPORT MEDIA

By

B Gastrin L O Kallings and A Marcetic

Received 6 vi 68

During the past decades numerous attempts have been made to find some means of maintaining the viability of sensitive microorganisms while being transported to the laboratory. Much of the earlier experiences are derived from attempts to improve gonorrhoeal diagnosis (*Peter & Steffen* 1943 *Reyman* 1944).

The first actual transportation medium for gonococci was made by Stuart (1959). Oxidation was prevented by the addition of thioglycollic acid. To inhibit growth of irrelevant bacteria all nutritive substrates were eliminated. Agar was added to preserve the anaerobic conditions by preventing agitation during transportation.

The presence of toxic substrates for gonococci in the agar and in the cotton of the swabs was demonstrated by *Ley & Mueller* (1946) and *Stuart et al* (1954) who showed that this effect could be eliminated by charcoal. *Moffet et al* (1948) recommended that the specimen swabs should be boiled in buffer and dried and dipped in a suspension of 1 per cent pulverized charcoal. *Ringertz* (1960) and *Reyn* (1960) confirmed that swabs treated in this way were superior to untreated ones for maintaining viability in gonococcal specimens in transit.

In addition to gonococci many other pathogenic microorganisms have been shown to survive in Stuart's medium for example meningococci *Shigella H pertussis* and anaerobic bacteria (*Cooper* 1957 *Stuart* 1956 1959). In general sensitive bacteria survived for 24 hours while less sensitive microorganisms survived a storage period of 2-3 weeks or longer at room temperature.

Möller (1966) described a transportation medium (VMG) which he found superior for demonstrating streptococci and anaerobic non sporulating bacteria in endodontic samples. In contrast to non nutrient transportation media as the Stuart medium it contained ingredients which might support growth and in addition a bacteriostatic agent.

None of the current media has been generally acknowledged as a transportation medium suitable for most bacteria in clinical and public health bacteriology. In general specimens are forwarded under conditions which do not prevent quantitative changes in the bacterial flora

The present investigation describes a new modification of Stuart medium designated SBL medium which maintained the flora unchanged for the great majority of the swab specimens. The survival time of some of the most important pathogens in clinical bacteriology has been compared in Stuart, AMG and SBL media.

TABLE 1
Bacteria and Plate Counting Media Used in the Experiments

		Strain	Counting media
1	Clostridium perfringens	CN-1493/1964 Type E (Wellcome)	Blood agar
2	Corynebacterium diphteriae	SBL-33/1948 *mitis* SBL-231/1957 *intermedius*	Loeffler serum agar
3	Escherichia coli	SBL-2635/1966 SBL C71/1966 O 111 B 4	Endo agar
4	Haemophilus parainfluenzae	SBL-3044/1966 SBL-4056/1966	Haematine serum agar
5	Haemophilus pertussis	NIH-44 122 (Bethesda) Danderyd Hospital-6728/1967	Bordet Gengou agar
6	Yersinia enterocolitica	Winblad 897 (Malmo)	*Blood agar*
7	Klebsiella	A-5054 type 1 (Copenhagen) *B-5055 type 2 (Copenhagen)*	Endo agar
8	*Listeria monocytogenes*	*Patersson 7973 type 1* *Patersson 5348 type 2*	Tryptose agar
9	Neisseria gonorrhoeae	SBL-19154/1967 SBL-29159/1967 SBL-114745/1967 SBL-114781/1967	Haematine serum agar
10	Neisseria meningitidis	SBL-4379/1966 2000 Centre Int (Lausanne)	Haematine serum agar
11	Pneumococcus	SBL-2292/1963 SBL-17519/1966	Blood agar
12	Proteus vulgaris	SBL-177/1927 SBL-17231/1966	*Desoxycholat citrat agar*
13	Pseudomonas aeruginosa	*SBL-1/864/1966* SBL-19283/1966	*Endo agar*
14	Salmonella	SBL-K566/1966 S typhimurium SBL-K1515/1966 S manhattan	Endo agar
15	Shigella	SBL-K203/1966 Sh sonnei Public Health Service Atlanta Sh flexneri type 1a/1953	Endo agar
16	Staphylococcus aureus	Oxford-209 London 5 phage type 80 81	*Blood agar*
17	*Streptococcus*	SBL-5-84/1954 group A Rockefeller Inst type 14-Griffith 1946 (New York)	*Tryptose agar*
18	Vibrio cholera	Inaba-47 (Fever Hospital Cairo) Ogawa-50 (Rainsford U S Navy Hospital Cairo)	Blood agar

Freshly isolated strains

MATERIAL AND METHODS

Bacterial Strains and Plate Counting Media

See Table 1

Transportation Media

Stuart medium (Modified by *Ringert* 1960) Bacto agar 10 g Thioglycollic acid 1 ml Na gl cerophosphate (20 per cent in water) 50 ml $CaCl_2$ solution (1 per cent in water) 10 ml Methylene blue solution (0 1 per cent in water) 2 ml Aq dest 930 ml pH 7 2

VMG (VMG II) medium Agar washed 7 g Bacto gelatine 5 g Tryptose 0 5 g Thiotone 0 5 g Cysteine hydrochloride 0 5 g Thioglycollic acid 0 5 ml Stock salt solution 100 ml Aq dest 900 ml

Stock salt solution Then Imercuric acetate 0 05 g $CaCl_2$ 6 H_2O 2 4 g KCl 4 2 g NaCl 10 g $MgSO_4$ 7 H_2O 1 g Sodium glycerophosphate 100 g Methylene blue 0 03 g Aq dest ad 1000 ml pH 7 5

SBL medium Bacto agar 8–10 g Thioglycollic acid 0 5 ml Sodium glycerophosphate 10 g $CaCl_2$ (1 per cent in water) 10 ml Cysteine hydrochloride 0 075 g Methylene blue (0 1 per cent in water) 2 ml Aq dest 930 ml rh = <0 01 pH 2 gel strength 25 g

The transportation kit is the same as that described by *Kallings* (1968) and *Castrin & Kallings* (1968) with the transportation media enclosed in nitrogen filled glass ampoules which were opened immediately before use

The swabs were ordinary cotton tipped wooden sticks which were boiled in Sørensen's buffer pH 4 treated with charcoal packed in plastic envelopes and sterilized by 4 5 Mrad of gamma radiation

Performance of the test The bacterial suspensions for inoculation of the swabs were made by growing the test microorganisms for 20 hours on agar media The bacteria were washed off the agar surface with phosphate buffered saline (PBS) The suspension was adjusted to contain 7–10 × 10^8 cells per ml and 0 1 ml aliquots were transferred to small tubes The swabs were left in these tubes until all the bacterial suspension was absorbed Afterwards the swabs were placed in ampoules containing the different transportation media and stored at room temperature in the dark for different times up to 120 hours

After the storage time the swabs were removed from the ampoules and placed in tubes with 5 ml PBS The tubes were shaken mechanically (50 cycles/sec) for approximately 20 sec The number of surviving bacteria in the PBS suspension was counted by the plate surface dilution method

This method for recovering bacteria from the swabs after they had been stored for one hour in the transportation medium resulted in a loss of 1–2 logs per swabs for the different bacteria For comparative purposes the number of bacteria which could be recovered from the swabs after one hour storage in the respective media was used as the reference value This value was expressed as the number of bacteria per ml PBS in which the swabs were shaken All other results are presented in the same manner

RESULTS

The change in the number of viable bacteria after different storage times for the various types of bacteria and transportation media examined is shown in Figs 1–3 The values are given as the average for one to four strains of the respective bacteria each strain was tested in dublicate

Group A streptococci and *Staphylococcus aureus* survived 120 hours in the different transportation media For Stuart and SBL media the bacterial count was almost unchanged after 120 hours On the other hand about 1 4–1 9 logs increase occurred in the VMG medium during the same time

The gonococci and meningococci count decreased continuously about 4 logs in 120 hours in all media during the storage time

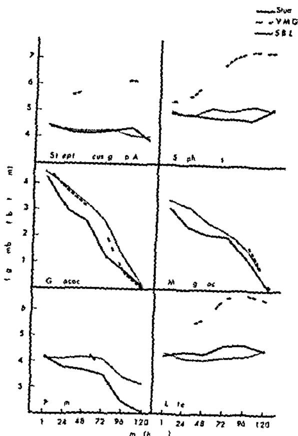


Fig 1

The change in bacterial number expressed in $\log_{10}$ after storage on charcoal treated swabs in 3 different transportation media for different times (1 120 hours) at room temperature

The pneumococci count in VMG and SBL media was almost unchanged after 72 hours but further there was a continuous decrease which after 120 hours amounted to about 1 log. In Stuart medium the decrease was more than 2 logs in 120 hours

The *Listeria monocytogenes* count remained approximately unchanged in Stuart s and SBL media after 120 hours while an increase of 1 9 logs occurred in VMG medium (Fig 1)

Haemophilus pertussis decreased by about 5 logs in 96 hours in Stuart s and VMG media against 1 5 logs in SBL medium The bacterial count was almost unchanged after 72 hours in SBL medium while a decrease of 2 3–3 0 logs occurred in Stuart s and in VMG media (Fig 2)

Haemophilus parainfluenzae showed a continuous decrease in SBL and Stuart s media amounting to 0 5–0 8 log after 120 hours However there occurred an increase of 1 2 logs after the same time in VMG medium

Haemophilus influenzae Three freshly isolated strains of *H influ*

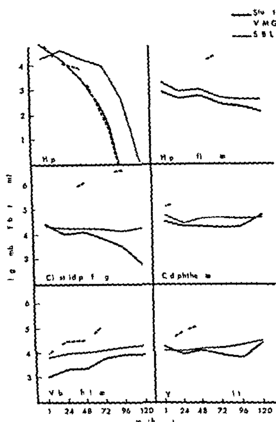


Fig 2
The change in bacterial number expressed in $\log_{10}$ after storage on charcoal treated swabs in 3 different transportation media for different time (1–120 hours) at room temperature

en ae were later included in the present investigation They were found to be more labile than Haemophilus para influenzae After 120 hours for SBL and Stuart media the decrease was about 2 log and for VMG medium 0 4 log

The Clostridium perfringens count did not change after 120 hours in SBL medium while it was reduced by 1 3 logs in Stuart s and increased by 1 2 logs in VMG during the same time

No major change in the bacterial count of *Corynebacterium diphteriae* occurred after 120 hours in any medium

The number of *Vibrio cholera* increased less than 0 8 log in 120 hours in SBL and Stuart s media while an increase of 1 7 log occurred in VMG

The *Yersinia enterocolitica* count was almost unchanged in SBL and Stuart s media after 120 hours while it increased by 1 4 log in VMG (Fig 2)

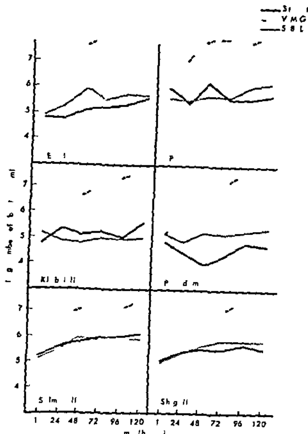


Fig 3

The change in bacterial number expressed in log after storage on charcoal treated swabs in 3 different transportation media for different times (1–120 hours) at room temperature

The gram negative rods belonging to *Escherichia Proteus Klebsiella Pseudomonas Salmonella* and *Shigella* behaved similarly during a storage time of 120 hours All these bacteria increased by 1 4–2 5 logs in VMG In Stuart s and SBL media they survived with a change of less than 1 log (Fig 3)

In order to simplify the evaluation of the results the bacteria were *arranged according to their survival capacity in SBL transportation* medium The survival capacity was measured after 72 hours storage in the transportation media and expressed as the log difference between the bacterial count after 1 hour and 72 hours (Fig 4) The results for Stuart and VMG media are given for comparison

The figure shows as expected that the pathogenic *Neisseria* and *H pertussis* were the most sensitive ones The difference in survival between many strains belonging to other bacterial species was so small that their order of arrangement may be connected with errors in the

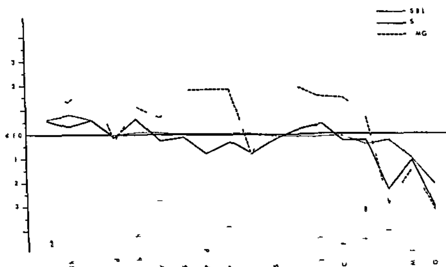


Fig 4

The investigated bacteria arranged in sequence according to their change in number after storage for 3 days at room temperature on charcoal treated swabs in SBL transportation medium The results from Stuart s and VMG media also are given for comparison

method or with individual variation in resistance of strains within the same species

After 72 hours none of the bacteria investigated increased in number by more than 0 8 log in the SBL and Stuart s media On the other hand 11 of the investigated strains had increased by more than 1 log in VMG medium

DISCUSSION

A transportation medium ought to be able to maintain all sensitive microorganisms viable but at the same time prevent growth of other bacteria which may be present in a sample The organisms should be preserved at the same level and in the same proportion they had at the sampling To fulfil these requirements the medium should have the following properties low nutrient content no inhibiting factors low oxidation reduction potential and a physiological pH The purity of the ingredients were found to be essential

Maline & Finn (1951) and *Moller* (1966) have shown that sodium thioglycollate is toxic for many microorganisms therefore the amount of this substance was reduced in the SBL medium as compared to the Stuart medium A favourable effect was obtained with a large number of tested microorganisms when a small amount of cysteine hydrochloride replaced part of sodium thioglycollate as reducing agent Earlier investigators have tested higher amounts with varying results (*Bo r t al* 1931 *Gould et al* 1944 and *Moller* 1966) In the experiments [illegible]

tration of 25 mg cysteine HCl per 1000 ml was found superior to higher concentrations

The concentration of calcium chloride used was in conformity with concentration used by recent investigators (*Stuart* 1959)

The use of sodium glycerophosphate as buffer was based on Stuart's report (1959) that this salt was superior to the inorganic phosphate when CaCl was used However some microorganisms metabolize glycerophosphate In such case the transportation medium may promote bacterial growth (*Cary & Blair* 1964)

The SBL medium contained 8–10 g washed agar per 1000 ml chlorine free distilled water The precise amount of agar was chosen according to measurement of the gel strength (*Jonsson* 1967)

Barlow et al (1955) have used resazurin as an Eh indicator However *Stuart* (1959) pointed out that methylene blue is decidedly better being more sensitive It reacts at Eh + 0 01 in contrast to resazurin which reacts at Eh + 0 05 Therefore methylene blue is used in the SBL transportation medium as well as in the others

Most previous investigations on the survival of bacteria in clinical specimens in transit have been designed to see whether there was any bacterial growth demonstrable after different periods of time Obviously the demonstration of survivors was dependent not only on the preserving effect of the transportation medium but also on the initial bacterial level and the efficiency of the culturing technique to detect small numbers of the different organisms As these factors may vary considerably from experiment to experiment and with the different organisms tested the general application of the results was uncertain In the present experiments therefore the factors of inactivation or multiplication have been determined by survival or growth curves based on viable cell counts to admit comparison between different investigations and bacterial species

In our study the inactivation for two out of the three species found to be most labile gonococci and meningococci amounted to between 1 1–1 8 logs in 72 hours when they were stored in the SBL medium at room temperature Consequently cultures containing a great number of cells can be expected to survive for a considerable time Actually the SBL medium has been routinely used to preserve gonococci strains for two weeks or more in the refrigerator The third labile organism *H pertussis* decreased only by 0 2 log in 72 hours at room temperature in the same medium offering greatly improved diagnostic possibilities The inactivation factor of this organism in Stuart's medium was 2 3 logs in 72 hours which still is probably a much better result than could be achieved by many current methods

The results revealed that the Stuart and SBL media also were suitable for the transportation of other clinically important pathogens such as the gram positive pyogenic cocci Further these media prevented the growth of the gram negative rods e g *Klebsiella Proteus* and *Pseudo*

monas which use to cause problems by overgrowing labile and fastidious organisms. The epidemiologically important pathogens tested belonging to the *Salmonella*, *Shigella* and *Vibrio* genuses as well as *Corynebacterium diphteriae* were found to survive without any greater change in the viable count.

The third medium tested, the VMG medium, offered as good survival capacity as the other two media but promoted growth of most bacteria examined. This growth promoting effect is according to *Moller* (1966) partly due to restoration of the reproductive capacity. It must be pointed out that VMG medium is made for transportation of endodontic samples. In this work the group of anaerobic non sporulating bacteria has not been studied. A multiplication during transportation may seem advantageous for the detection of specific pathogens as for instance *Salmonella* and *Corynebacteria* but is generally combined with the multiplication of other bacteria in the specimen interferring with the diagnostic efforts.

The comparison between the three transportation media revealed that the least increase or decrease in the count of the tested bacteria appeared in the SBL medium. This medium therefore seems suitable to use as a common transportation medium in different kinds of bacteriological examinations.

SUMMARY

A quantitative approach to diagnostic bacteriology necessitates measures which maintain the bacterial counts unchanged during the transportation of the specimens to the laboratory, preserving the viability of labile organisms and preventing the multiplication of the rest. For swab specimens this objective can be accomplished by using a suitable transportation medium, e.g. the Stuart's medium for gonococci.

A modified Stuart's medium (SBL) intended for the transport of most kinds of swab specimens has been compared to the Stuart medium and a third medium based on other principles. The media were kept in nitrogen filled glass ampoules. Nineteen different bacterial species common in clinical bacteriology have been used as test organisms. The most favourable results were obtained with the modified medium, thus as labile an organism as *Haemophilus pertussis* for example decreased by only 0.2 log after 72 hours at room temperature and no bacteria tested increased by more than about one log in number during that time.

REFERENCES

1. *B lou J I, Yamak m Y & Cavanaugh D C*: A comparis n t l f transport media. Jap J med Sci Biol 8 171–174 19 5
2. *Boor A K & Miller C I*: Growth requirements of gonococcus protein medium. Arch Path 12 137–139 1931
3. *Cary C S & Blair B E*: New transport medium for shipment mens I Fecal specimens. J Bact 88 96 98 1964
4. *Cooper G N*: The prolonged survival of upper respiratory tract pathogens on swabs. J Clin Path 10 26–230 19 7

5 *Castrin B & Kallings L O* Improved methods for gonococcal sampling and examination in large scale Acta path microbi l scandinav 74 362-370 1968
6 *Gould C R Kane W I & Mueller H J* On the growth requirements of Neisseria gonorrhoeae J Bact 47 287-292 1944
7 *Johnsson F* Personal communication 1967
8 *Kallings L O* Transport of bacteriological specimens Biotechnol Bioeng 10 399-401 1968
9 *Ley H L & Mueller J H* On the isolation from agar of an inhibitor for Neisseria gonorrhoeae J Bact 52 453-460 1946
10 *Moffet M Young J L & Stuart R D* Centralized gonococcus culture for dispersed clinics Brit Med J 2 421-424 1948
11 *Möller J R A* Microbiological examination of root canals and periapical tissues of human teeth Akademif rlaget-Coteb rg 1966
12 *Lever R L & Steffen C I* Improved method of transportation of material from gonococcal infections J Lab Clin Med 28 1121-1126 1943
13 *Reymann F* Cultivation of gonococci as diagnostic method in gonorrhoeae in women Acta derm venerol Stockholm 25 9 23 1944
14 *Reyn A Korner B & Bentzon M W* Transportation of material for the culture of the gonococcus Brit J vener Dis 36 243-256 1960
15 *Ringert O* A modified Stuart medium for the transport of gonococcal specimens Acta path microbiol scandinav 48 105 112 1960
16 *Stuart R D* The diagnosis and control of gonorrhoea by bacteriological cultures With a preliminary report on a new method for transporting clinical material Glasg Med J 27 131-142 1946
17 *Stuart R D Toshach R S & Patsula M T* The problem of transport of specimens for culture of gonococci Canad J Publ Hlth 45 73-83 1954
18 *Stuart R D* Transport problems in public health bacteriology The use of transport media and other devices to maintain the viability of bacteria in specimens Canad J Publ Hlth 47 114-122 1956
19 *Stuart R D* Transport medium for specimens in public health bacteriology Publ Hlth Rep (Wash) 74 431-438 1959

Acta path. microbiol. scandinav. 74: 381–390, 1968

Statens Seruminstitut Regional Laboratory (Head: K. Siboni)
The City and County Hospital, Odense

THE SENSITIVITY TO METHICILLIN AND PENICILLIN OF METHICILLIN RESISTANT STRAINS OF STAPHYLOCOCCUS AUREUS

By

KNUD SIBONI and LONE KROGH

Received 3.v.68

The increasing frequency of *Staphylococcus aureus* resistant to methicillin in hospitals in France (*Chabbert & Pillet* 1967), England (*Jevons et al.* 1963, *Stewart & Holt* 1963) and Denmark (*Eriksen & Erichsen* 1964) has been depriving physicians of their most efficient drug in the treatment of severe staphylococcal infections. The nature of this naturally occurring resistance to methicillin has been discussed by *Barber* (1964) who found the methicillin resistant staphylococci protoplast like. Both *Barber* and *Eriksen & Erichsen* (1964) have described the inoculum effect of these bacteria towards methicillin, i.e. that resistance increases with the size of the inoculum. Eriksen & Erichsen ascribed less importance to a penicillinase independent resistance of low degree and more to the production of penicillinase. *Seligmann* (1966), however, who worked with one of Eriksen's strains, could *in vitro* select stable variants with high resistance and ascribed therefore more importance to a hypothesis of high step mutations, their frequency being c. 10^{-4}, while the rest of the population had only slightly decreased sensitivity. *Chabbert* (1967) avoided penicillinase production by treating the cells with acriflavine and could support Seligmann's description. Most cells in the population being rather sensitive, treatment with methicillin might be regarded feasible but proved unsuccessful.

The cephalosporins cephaloridine and cephalothin are believed to have the same kind of effect on staphylococci as methicillin (*Barber & Waterworth* 1964), the effect per weight unit of cephaloridine being eight times that of methicillin (*Chabbert & Pillet* 1967) both on methicillin sensitive and on methicillin resistant strains of *Staphylococcus aureus*. *In vitro* *Bulger* (1967) found synergistic effect of cephalothin plus kanamycin on methicillin resistant staphylococci but results in patients have been doubtful (*Chabbert* 1967).

We are grateful to *Michael Weis Bentzon*, actuary, Biostatistical Department [illegible] for checking through the statistical calculations.

5 *Castrin B* & *Kallings I O* Improved methods for gonococcal sampling and examination in large scale Acta path microbiol scandinav 74 362 370 1968
6 *Gould C R* *Kane W I* & *Mueller H J* On the growth requirements of Neisseria gonorrhoeae J Bact 47 287–292 1944
7 *Johnsson F* Personal communication 1967
8 *Kallings L O* Transport of bacteriological specimens Biotechnol Bioeng *10* 399–401 1968
9 *Ley H L* & *Mueller J H* On the isolation from agar of an inhibitor for Neisseria gonorrhoeae J Bact *52* 453–460 1946
10 *Moffet M* *Young J I* & *Stuart R D* Centralized gonococcus culture for dispersed clinics Brit Med J 2 421–424 1948
11 *Möller J R A* Microbiological examination of root canals and periapical tissues of human teeth Akademiforlaget–Goteborg 1966
12 *Peizer R L* & *Steffen G L* Improved method of transportation of material from gonococcal infections J Lab Clin Med 28 1121–1126 1943
13 *Reymann F* Cultivation of gonococci as diagnostic method in gonorrhoeae in women Acta derm venerol Stockholm 25 9–28 1944
14 *Reyn A* *Korner B* & *Bent on M W* Transportation of material for the culture of the gonococcus Brit J vener Dis *36* 243–256 1960
15 *Ringert O* A modified Stuart medium for the transport of gonococcal specimens Acta path microbiol scandinav *48* 105–112 1960
16 *Stuart R D* The diagnosis and control of gonorrhoea by bacteriological cultures With a preliminary report on a new method for transporting clinical material Glasg Med J 27 131–142 1946
17 *Stuart R D* *Toshach R S* & *Patsula M T* The problem of transport of specimens for culture of gonococci Canad J Publ Hlth *45* 73 83 1954
18 *Stuart R D* Transport problems in public health bacteriology The use of *transport media and other devices to maintain the viability* of bacteria in specimens Canad J Publ Hlth 47 114–122 1956
19 *Stuart R D* Transport medium for specimens in public health bacteriology Publ Hlth Rep (Wash) 74 431–438 1959

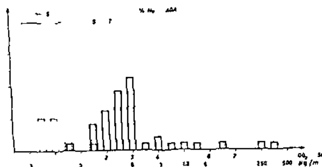


Fig 1

Methicillin sensitivity of *Staphylococcus aureus*
Distribution of IC50 read after 48 hrs

Table 1 shows that while the two parallel rows of tubes usually gave the same end point with the methicillin sensitive strains skew titres were significantly more common with the methicillin resistant strains A comparison with the results from tests with another species *Flavobacterium meningosepticum* (*Olsen* 1967) shows that this rod behaved against twelve antibiotics and among these streptomycin as methicillin sensitive strains of *Staphylococcus aureus* did to methicillin and differed from the methicillin resistant strains in their behaviour to methicillin These skew titres of the methicillin resistant strains are best explained by a big difference between the sensitivities of the most resistant bacterium of the two inocula of parallel tubes i e

TABLE 1

Determination of IC50 of Staphylococcus aureus against Methicillin and of Flavobacterium meningosepticum against Various Antibiotics

	Staphylococcus aureus methicillin sensitive	*Staphylococcus aureus* methicillin resistant	*Flavobacterium meningosepticum*
Two rows of tubes with the same end point	31	35	256
a difference of 1 tube	9 }	30 }	55 }
a difference of 2 tubes	0 }	5 }	3 }
Number of tests	40	70	314
	$\chi^2 = 8.02$ $f = 1$ $0.001 < P < 0.005$		$\chi^2 = 31.1$ $f = 1$ $P < 0.0005$
Average difference	$\frac{9}{40} = 0.225$	$\frac{40}{70} = 0.571$	$\frac{61}{314} = 0.194$
Mean ratio of the sensitivities of the most resistant bacterium in the two parallel tubes	1.17	1.49	1.14

Each end point was determined by two parallel rows of tubes containing two fold dilutions of antibiotic in infusion broth Inoculum $10^{3.9}$ bacteria per tube

by a pronounced heterogeneity of the sensitivity of the individual bacteria

The standard deviation of IC50 in repeated experiments with eleven strains was found to be $s_R = 0.95$ log $f = 11$ ($s_R = 0.90$) The 95 per cent level will permit a variation between two determinations of the same strain of $2.2\sqrt{2}\, s_R = 3.1$ log. This standard deviation may therefore account for the variation of log IC50 between 1.0 and 3.5 in Fig 1 but not beyond these limits

If the two parallel rows of tubes used in each sensitivity test are regarded as repeated determinations within the same experiment (Table 1) the variance within the experiment is found to be $s_E^2 = \frac{30+5\times4}{2\times70} = 0.36$ and the variance of the average of two rows is 0.18 log

Observations in Patients

The great difficulties in the antibiotic treatment of patients with bacteraemia due to methicillin resistant staphylococci are demonstrated in Fig 2 (patient 1)

Patient 1 a 41 year old male was operated upon on the 10th of February after a perforation of the colon diagnosis ulcerous colitis He was re operated on the 20th of March for bleeding duodenal ulcer He eventually recovered from the staphylococcal bacteraemia during treatment with cephaloridine

It was therefore hoped that big doses of methicillin would prove efficient against infections due to methicillin resistant staphylococci

Patient 2 an 84 year old female was operated upon on the 12th of May for gangrenous appendicitis The staphylococcal focus was not diagnosed pus from the peritoneum did not contain *Staphylococcus aureus* but the focus may have been in the lungs Treatment with methicillin apparently reduced the bacteraemia (Fig 3) but after three weeks of methicillin a few staphylococci could still be isolated from the blood and these were 24 times as resistant as the ones first isolated The patient died

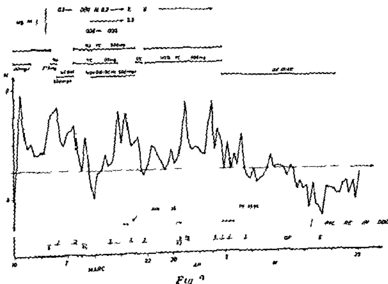


Fig 2

Fig 3
Patient 2

Fig 4
Patient 3

In the treatment of the next patients nos 3 and 4 are therefore relied on the experiences gained in the case of patient 1 and the treatment was van omycin followed by methicill n

Patient 3 a 5? year old male with chronic bronchitis and pneumonia H w cured bacteriologically and clinicall (Fig 4)

Patient 4 a 14 year old male with ulcerous endocarditis who die l A t days of vancomycin the intensity of the b cteraemia was reduced a in p n s 1 and 3 the reduction being evident in that half of the tubes inoculated th l o d did not give growth of *Staphylococcus aureus*

Population Analysis

Strains of *Staphylococcus aureus* isolated from the blood c t nt 2 before and during treatment with methicillin (Fig 3) ver tried against methicillin Fig 5 shows that all bacteria were resist n ?(4)

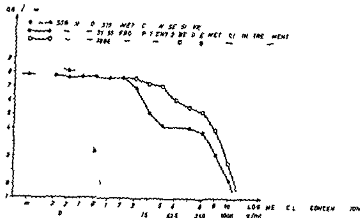


Fig 5

Population analysis of methicillin sensitivity of *Staphylococcus aureus* (Patient 2

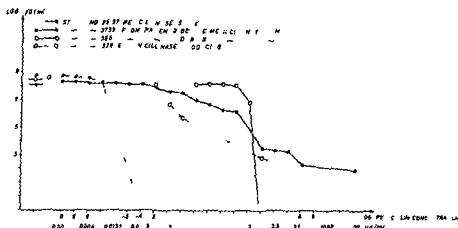


Fig 6

Population analysis of penicillin sensitivity of *Staphylococcus aureus* (Patient 2

μg/ml before treatment with methicillin (strain 37 593) and the majority were sensitive to 2 (32) μg/ml. More resistant bacteria had a frequency of c. 10^{-4}. During treatment with methicillin the frequency of highly resistant bacteria increased (strain 38 862).

Fig 6 shows the high grade inoculum effect of this penicillinase producing staphylococcus before treatment with methicillin (strain 37 593). During treatment with methicillin the strain (38 862) became dominated by bacteria with a high resistance to penicillin ($>2^3(8)$ μg/ml) but with no inoculum effect. In Fig 7 analysis against penicillin of penicillinase negative variants of the two strains reveals curves analogous to those obtained with methicillin (Fig 5).

Strains isolated from the blood of patient 1 before (strain 34 635) and during (strain 35 962) treatment with penicillin (Fig 2) were

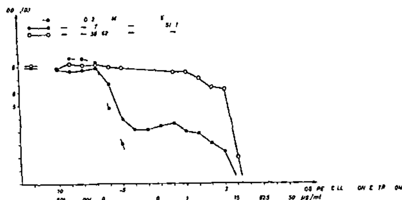


Fig 7

Population analysis of penicillin sensitivity in penicillinase negative strains of *Staphylococcus aureus* (Patient 2)

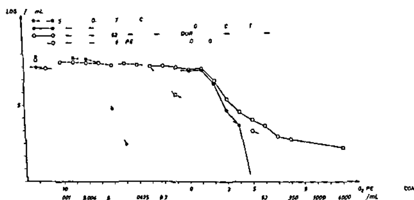


Fig 8

Population analysis of penicillin sensitivity of *Staphylococcus aureus* (Patient 1)

tried against penicillin Fig 8 shows that most of the cells of these two strains were more resistant to penicillin than those of the methicillin sensitive control strains 25 157 and 1529/67 and that treatment with penicillin heightened the inoculum effect Analysis with methicillin revealed curves congruous to those obtained with strains 37 593 and 38 862 from patient 2 (Fig 5 and Table 2)

Table 2 shows the results of population analysis of methicillin sensitivity in *Staphylococcus aureus* isolated from patients 1 3 and 4 before and during treatment with vancomycin This treatment of the patients did not make the staphylococci more susceptible to methicillin and those of patient 1 who had penicillin at the same time as vancomycin became more resistant to methicillin

TABLE 2

Population Analysis of Methicillin Sensitivity in Six Strains of Staphylococcus aureus *Isolated from the Blood of Three Patients before and during Treatment with Vancomycin*

Patient	1		3		4	
Strain no	34 635	35 962	3505/67	4154/67	6320/67	6501/67
Number of bacteria per 0 1 ml	135×10^{6}	159×10	196×10^{6}	150×10	282×10^{6}	100×10^{6}
resistant to						
2 µg/ml	68×10^{4}	81×10^{6}	103×10^{6}	79×10^{6}	145×10^{6}	60×10^{6}
4 µg/ml	2×10^{4}	45×10^{6}	100×10^{6}	41×10	89×10^{6}	34×10^{6}
7 9 µg/ml	72×10	15×10	57×10^{6}	29×10^{6}	600×10^{4}	25×10^{6}
15 7 µg/ml	40×10	180×10^{4}	19×10^{6}	4×10^{6}	5×10^{4}	598×10^{4}
31 3 µg/ml	36×10	75×10^{4}	58×10^{4}	22×10^{4}	38×10	2×10^{4}
62 5 µg/ml	18×10	12×10^{4}	1×10^{4}	151×10	31×10	114×10
125 µg/ml	20×10	221×10	90×10	41×10	14×10	20×10
250 µg/ml	260	30×10	248	222	146	221
500 µg/ml	198	260	152	122	76	78
Frequency of bacteria resistant to >15 7 µg per ml	03×10^{-4}	47×10^{-4}	30×10^{-4}	15×10^{-4}	01×10^{-4}	20×10^{-4}
End point with tube dilution method in broth						
log IC50 (24 hs)	2 5	1 5	1 0	0 0	2 0	1 0
log IC50 (48 hs)	4 0	3 5	4 0	0 5	4 5	3 0
in broth containing 7 5 per cent NaCl						
log IC50 (24 hs)	4 5	1 5	5 5	4 5	4 5	4 5
log IC50 (48 hs)	5 5	3 5	6 5	6 5	6 5	6 0

DISCUSSION

Sensitivity tests of the methicillin resistant staphylococci (Table 1) have shown the great variability of the end points in comparison to those of the methicillin sensitive strains under conditions where this could not be due to different sizes of the inocula This variability is therefore best explained by a high frequency of highly resistant mutants (*Welsch* 1949 *Seligmann* 1966) Population analysis (Table 2 and Fig 5) indicated the same frequency of 10^{-4} as Seligmann suggested

Methicillin treatment *in vivo* resulted in the selection of a stable variant (mutant) highly resistant to methicillin and with an inoculum independent resistance to penicillin (Fig 5 and 6) This is a parallel to *Seligmann s* experiments *in vitro* and to *Chabbert s* (1967) experiences obtained in *in vivo* and it warns against methicillin as the sole drug in the treatment of infections due to methicillin resistant staphylococci

After treatment with vancomycin which perhaps reduced the number of bacteria in the blood to about one per ml i e about 10^{4} in the total extracellular volume of fluid the frequency *in vitro* of highly resistant bacteria was unaltered (Table 2) This again fits the hypothesis of the mutational origin of the highly resistant variants But in addition the small number of bacteria highly resistant to methicillin in *the blood of*

patients with low grade bacteraemia could explain the possible effect of cephaloridine in patient 1 and of methicillin in patient 3 when given after a course of vancomycin This suggests the use first of an anti biotic which can reduce the intensity of the bacteraemia such as vanco mycin or kanamycin and then after 4–6 days the addition of a methi cillin like drug may give the effect known from the methicillin sensitive strains Here one of the cephalosporins may be chosen on account of their greater effect per weight unit

The last rows in Table 2 show log IC50 of the same strains in the tube dilution test The end points were an average of 3 log higher read after 48 hs when broth with 7 5 per cent sodium chloride was used This is consistent with the results obtained by *Chabbert & Pillet* (1967) and even if these results corresponding to an osmotic pressure of more than eight times that of blood are not relevant as regards the effect of methicillin on staphylococci in the circulating blood they may well be so as regards the effect in the focus Patient 1 who was repeatedly operated upon and thereby had abscesses in the abdomen removed re covered Patient 3 who had only pulmonary foci was bacteriologically cured but patient 4 with the endocardial focus died *Ubi pus ibi evacua* is more relevant than ever with the infections due to the methicillin resistant staphylococci

Findings in patient 1 (Fig 8 and Table 2) and patient 2 (Figs 5 and 6) have shown that the methicillin resistant strains of *Staphylo coccus aureus* are favoured not only by the ability to produce penicillin ase as are other penicillin resistant staphylococci but also by the abi lity to throw variants (mutants) with a high inoculum independent resistance to penicillin (Figs 6 and 7) 500–1000 times that of a peni cillin sensitive strain and at the same time highly resistant to methi cillin (Fig 5) This in addition to the insensitivity of the methicillin resistant staphylococci to erythromycin and most often to chloramphe nicol may explain why they have taken over the place as dominants from the strain resistant only to penicillin streptomycin and tetracyclin (*Siboni & Digmann Poulsen* 1968) And as penicillin has been used far more than chloramphenicol erythromycin and methicillin the use of penicillin may have been the most important selective force

SUMMARY

The skew titres obtained in tube dilution tests of the methicillin sen sitivity of methicillin resistant staphylococci demonstrated the hetero geneity of the population and are in accordance with the high step mutation hypothesis

Treatment with methicillin of a patient with bacteraemia due to a methicillin resistant *Staphylococcus aureus* resulted in the selection of a stable variant with high resistance to methicillin and an inoculum independent resistance to penicillin In another case treatment with penicillin seemed to induce penicillinase production

This warns against penicillin and methicillin as the sole drugs in the treatment of bacteraemia with methicillin resistant staphylococci and it points to both penicillin and methicillin as forces in the selection of the methicillin resistant strains of *Staphylococcus aureus*

If the staphylococcal population is already reduced through the treatment with other drugs the number of highly resistant variants in the blood will be small and drugs with methicillin like effect may be beneficial This may be the background of cure in two cases described

REFERENCES

Barber M Naturally occurring methicillin resistant staphylococci J Gen Microbiol 35 183–90 1964

Barber M & Waterworth P M Penicillinase resistant penicillins and cephalosporins Brit Med J 2 344–49 1964

Bulger R J In vitro activity of cephalothin/kanamycin and methicillin/kanamycin combinations against methicillin resistant *Staphylococcus aureus* Lancet *1* 17–19 1967

Chabbert Y A Behaviour of methicillin heteroresistant staphylococci to cephaloridine Postgrad Med J suppl vol *43* 40–42 1967

Chabbert Y A & Pillet J Correlation between methicillin resistance and sero type in *Staphylococcus* Nature *213* 1137 1967

Eriksen K R & Erichsen I Resistance to methicillin isoxazolyl penicillins and cephalothin in *Staphylococcus aureus* Acta path microbiol scandinav *62* 255–75 1964

Jevons M P Coe A W & Parker M T Methicillin resistance in staphylococci Lancet *1* 904–07 1963

Mackie & McCartney Handbook of Bacteriology 10th ed Livingstone 1962 p 355

Olsen H An in vitro study of the antibiotic sensitivity of *Flavobacterium meningosepticum* Acta path microbiol scandinav *70* 601–12 1967

Perret C J Iodometric assay of penicillinase Nature *174* 1012–13 1954

Seligmann S J Methicillin resistant staphylococci Genetics of the minority population J gen Microbiol *42* 315–22 1966

Siboni K & Digmann Poulsen E The dominance of methicillin resistant staphylococci in a county hospital *Dan Med Bull 15* 161–65 1968

Stewart G T & Holt R J Evolution of natural resistance to the newer penicillins Brit med J *1* 308–11 1963

Vatra J J Development of resistance to novobiocin tetracyclin and a novobiocin tetracyclin combination in *Staphylococcus aureus* populations J Bact *93* 801–05 1967

Welsch M Variations individuelles de la sensibilité a la streptomycine dans les populations staphylococciques normales Comp rend Soc biol *143* 1141–44 1949

Acta path microbiol scandinav 74 391 396 1968

Department of Microbiology The Gade Institute and Medical Department A
School of Medicine University of Bergen Bergen Norway

BACTERIAL COUNTS IN URINE

1 *The Reliability of the Loop Technique*

By

J HALGEN O STRØM and B ØSTERVOLD

Received 9 ix 68

Determination of the number of bacteria in urine is a widely accepted method of distinguished contamination from true bacteriuria and is therefore a useful procedure in the diagnosis and treatment of urinary tract infections (2 4 5)

Bacterial counting was originally performed by the classic dilution pour plate technique or modifications of this method These procedures are however time consuming and expensive and therefore not convenient for clinical and epidemiological use

In 1960 *Hoeprich* described a more simple rapid method for quantitative urine culture (1) By means of standard volume calibrated bacteriological loops streak plate cultures were prepared by direct inoculation from the urine specimen There is however no general agreement about the reliability of this method

The present work deals with the volume and the variation in volume of urine transferred to the media by such calibrated loops and concerns especially the influence on the transferred volumes of the technique used

MATERIALS AND METHODS

Urine Physiological urines of different specific weights (spec w) were used The spec w was determined by urometer One sample (A) was made radioactive by adding a solution of Mercury²⁰³ Neohydrin (MKNE) (Institutt for Atomenergi Kjeller Norway) Another sample (B) of 1/10 radioactivity strength of the former sample was prepared by dilution (w/w) Two bacteriological loops were used They were manufactured by K A Rasmussen Hamar Norway from Pt and 10 per cent Ir on the specifications given by *Hoeprich* (1) to deliver 0 01 and 0 001 ml urine respectively to the culture plates by the streaking technique recommended (3)

Pipette A serological pipette (Silberbrand) of 1 ml (Graded 1/100) was used

Dishes Plain Petri plastic dishes diameter 55 mm and depth 7 mm which all contained an equal amount of agar forming a 3 mm thick layer Urine was transferred to several series of dishes by the pipette or by the loops

Transfer of urine Urine subsamples were obtained by immersing the loop in the urine sample either approximately horizontally (H) using it as a b ler or by dipping the loop vertically (V) into the urine The subsamples were transferred and spread on the agar by the streaking technique (3) Subsamples transferred by the pipette were spread on the agar by rocking the dishes gently

Weighing of urine The weight of the urine volumes delivered by the pipette was determined gravimetrically by Mettler scales (1 div = 0.1 mg) After a tenfold weighing of the same volume the measurement error of the weighing procedure expressed as the coefficient of variation (CV w) was calculated

Measurement of radioactivity The gamma activity (radioactivity) of the urine transferred to each Petri dish was recorded by means of a thallium activated sodium iodide crystal 3 × 2 in connected to a pulse height analyser and scaler The scintillation crystal and the probe were provided with an all round lead shielding of 2 cm thickness The window settings for the gamma ray energy was 260–310 kev The background rate was at the average 50 counts/min The dish was placed concentric on the top of the crystal The activity (counts/min) was taken as a measure for the amount of urine transferred

After counting the activity of one dish from each series of dishes 10 times the measurement error of the counting rate expressed as the coefficient of variation (CV c r) was determined In order to include the error which might result from minor differences in the position of the dish in the well the dish was removed from the well between each of the countings

Statistics The mean standard deviation (S D) and coefficient of variation (CV) were calculated by the commonly used methods The difference of means was calculated by the t test A 5 per cent (0 05) level of significance was used

RESULTS

a Reference Activity

The measurement of the volumes transferred by the loops was based on comparison of the radioactivity of loop transferred urine with a reference activity representing a known urine volume The reference activity was determined by the following method

By a tenfold replication 0 10 ml urine of spec w 1003 (sample B) was delivered by the 1 0 pipette and weighed The weights were directly converted into volumes and the mean volume delivered was found to be 0 10521 ml with a CV of 1 93 per cent This CV is taken as the CV for the pipetting error of the volume transferred (CV v) as the CV w was found to be negligible (0 00057 per cent)

By the same pipette 0 10 ml (mean volume = 0 10521 ml) urine of the same sample (B) was transferred to each of 20 Petri dishes The radioactivity of the urine subsamples was determined The mean activity of the subsamples was used as a reference standard The CV for these determinations was 4 35 per cent This CV includes the combined effects of the variation in the volume delivered (CV v = 1 93 per cent) and of the measurement error of the counting rate (CV c r = 0 36 per cent) and in addition probably an effect of some variation due to differences in thickness of dishes and media The CV obtained is therefore an expression of the total variation in the activity (CV t) of the volumes transferred to the dishes

The determination of urine volumes by measurement of radioactivity gives a somewhat higher CV t than CV v and is thus encumbered with more errors than the weight method The errors are however relatively small and probably approximately constant in all measurements of radioactivity The CV t should therefore give a relatively good expression of the variation in transferred volume

b. *The Influence of Various Transferring Techniques*

After immersing the loop horizontally (H) or vertically (V) in urine of spec. w. 1003 (sample A), subsamples were transferred by both the 0.01 and 0.001 ml loops to four series of Petri dishes. The mean volumes transferred by the loops were calculated by comparing the mean radioactivity of each of the four series to the reference standard made for this urine (Table 1).

TABLE 1

The Mean Radioactivity, the Standard Deviation (S.D.) and the Coefficient of Variation (C.V. t) for the Activity of Dishes of Urine in Different Series. The Mean Urine Volume Transferred to Dishes by Pipette is Calculated by Weight Results, the Mean Volume Transferred by Loops by Measurement of Radioactivity

Calibrated vol. ml	Loop§ technique	No. of dishes	Radio-activity counts/min (mean)	S.D. counts/min	C.V. t %	C.V. c.r. %	Mean vol. transferred ml
Loop 0.001	H	19	4071	220.1	5.41	0.98	0.00213
	V	20	2456	133.2	5.42	1.76	0.00127
Loop 0.01	H	20	40496	5314.0	13.12	0.35	0.0209
	V	20	19218	2929.0	15.24	0.78	0.0099
Pipette 0.10		20	20584	895.8	4.35	0.36	0.1051

§ H and V: The urine specimen obtained by immersing the loop in the urine sample horizontally (H) and vertically (V) respectively.
C.V. c.r.: Coefficient of variation for the counting rate.

The results show that the volume transferred by the loop to the dish is determined by the technique used. They show moreover that when the urine is obtained by holding the loop vertically the volume transferred closely approximates the calibrated volume for each loop. Holding the loop horizontally gives a volume almost twice as great as the calibrated volume. The use of the latter technique necessitates the introduction of a correction factor. Irrespective of the technique used the C.V. t is approximately the same. The variation in transferred volume should therefore also be about the same. The variation in the volume transferred by the smallest loop is approximately equal to that in pipette transfer, the variation in those transferred by the largest loop being considerably greater.

c. *The Influence of Differences in the Spec. W. of Urine*

The influence on the transferred volumes of differences in the spec. w. of the urine was recorded. Urine of spec. w. 1003 and of spec. w. 1023 was used, a reference standard being made of both. By both techniques of obtaining the urine (H and V), a loop full of urine was transferred by both loops to series of Petri dishes. The mean volumes

transferred were calculated as described earlier the results being given in Table 2 The results show that there is little difference between the transferred volumes of urine of spec w 1003 and 1023 and that the difference between the mean volumes is not significant

TABLE 2

The Mean Volumes of Urine with Different Specific Weights Transferred to Dishes by Loops Using Different Techniques The Results Are Based on the Mean Radioactivity of Several Series of Dishes See Table I

Loop calibrated vol/ml	Urine spec w	Loop technique	No of dishes	Transferred vol (mean) ml	C V %
0.001	1003	H	19	0.00213	4.1
	1023	H	11	0.00211	6.72
	1003	V	20	0.00127	5.42
	1023	V	13	0.00142	9.79
0.01	1003	H	20	0.0209	13.1
	1023	H	13	0.0211	13.60
	1003	V	20	0.0099	15.24
	1023	V	12	0.0109	14.76

d *The Influence of Wear and Tear of the Loops*

The loops used for the experiments were three years old and had been used daily for at least one year The results might therefore have been influenced by some wear and tear of the loops Control measurements of the light opening of the loops and the diameter of the wires used for shanks and loops were therefore made by the manufacturer The measurements obtained with 1/1000 mm accuracy did not deviate 1/100 mm from those recorded at delivery or from the measurements of new loops

DISCUSSION

For quantitative bacterial counts in urine it is important to know the exact volumes of urine transferred to the media The loop technique has been considered to be a crude method giving a great variation in volumes transferred and thus results of little reliability

The volume transferred to the media by loops is far more difficult to determine than the contents of the loops The weight method was not found to be feasible due to a steady evaporation from the media leading to considerable inaccuracy in weight results The spectrophotometric method was also found to be unsuitable

As the present results show the chosen method is also encumbered with some errors and is thus not ideal However the present method permits an exact determination of the mean volume of urine transferred by loops The variation in transferred volumes is however

difficult to determine exactly by this method The best obtained expression for the variation in volume is the C V t which is an expression for the variation in radioactivity The real variation in volume is no doubt somewhat lower than that expressed by the C V t values presented Thus the C V v was 1 93 per cent by use of pipette the corresponding C V t being 4 5 per cent Nevertheless the method gives valuable information about the reliability of the loop technique for the determination of the number of bacteria in urine

The present findings show that the technique used in obtaining urine by a loop determines the volumes transferred It is therefore important to know for which technique the loop is calibrated and which technique is used in experiments where loops are employed This fact does not seem to have been considered in the earlier description of the loop technique (1) As the technique seems to be critical for the volumes transferred by the loop there will probably be a certain difference in volume related to the individual techniques employed by different technicians These differences have not been examined in this study as all the work was done by one experienced technician Furthermore the volumes transferred may to a certain degree depend on the physical properties of the media used It is therefore recommended that bacterial counts made by the loop technique should be performed by one technician in each laboratory and that the volumes transferred should be determined for the loops and the media used If this is done as in the present work the bacterial counts may be corrected to the real volume of urine transferred Furthermore the variation in delivered volume will be relatively small especially the variation in that transferred by the smallest (0 001 ml) loop It is this loop that is most important in separation of contamination from true bacteriuria

In addition there is a possibility that wear and tear of the loops may change the volumes transferred Although the loops used for these experiments had not changed after being used for 3 years such a possibility still exists and the calibration should therefore be repeated from time to time

Another objection to the use of a loop technique has been that the volumes transferred may vary with the viscosity and the specific weight of the urine In the present study the viscosity of the urines was not determined but there was no significant differences in transferred volume of urine when urines of different specific weights were used

The present experiments show that with the recommendations presented the loop method for quantitative urine culture is sufficiently accurate for clinical and epidemiological use

SUMMARY

The present work deals with the reliability of quantitative urine culture by the loop method of *Hoeprich* Urines of various specific weights

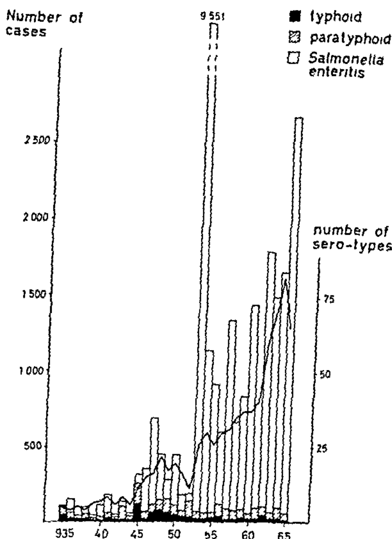


Fig 1

Number of cases and sero types of Salmonella in Sweden 1935–1965

participants (69 per cent) Totally *Salmonella* bacteria were isolated from 13 per cent of the tourists concerned in this study As this figure was obtained from groups of travellers among whom at least 1 case of *Salmonella infection was known to have occurred it does not imply* that 13 per cent of all tourists returning from non scandinavian countries are carriers of *Salmonella* On the other hand the probability of isolating *Salmonella* from the specimens was reduced by the circumstance that they were taken *on an average 3 4* weeks after the travellers *returned to Sweden* As would be expected a higher *proportion* of positive cultures was obtained from specimens collected soon after the return The magnitude of this reduction *may be calculated from Fig 3*

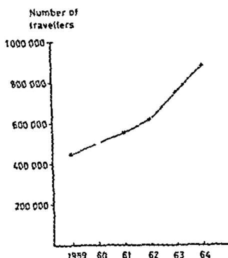


Fig 2

Number of Swedes visiting non Scandinavian countries 1959–1964

showing the duration of the carrier state in 466 clinical cases of *S typhi murium* infection in Vaggeryd Sweden 1962 (4) The curve indicates that approximately ¼ of the cases had ceased to excrete bacteria 3 weeks after the onset of illness and after 4 weeks nearly half of the number were negative

In the majority of cases only one specimen from each tourist was examined Judging from investigations made during the Vaggeryd out break this method of sampling meant that approximately 15 per cent escaped diagnosis

TABLE 1

Incidence of Salmonellosis in Travellers Participating in Conducted Tours

Country	Number of tours	Number of travellers	Number of travellers excreting Salmonella	Per cent positive cultures
Italy	28	2 362	239	12
Spain Mallorca Canary Island	16	866	44	6
Greece	8	644	111	18
Roumania	8	390	82	21
Jugoslavia	2	85	13	16
Israel	1	84	4	5
Germany	1	83	1	2
Austria	1	30	1	3
Germany Switzerland Italy	1	44	27	69
Netherlands Paris Mosel	1	69	1	2
Total	67	4 58	503	13

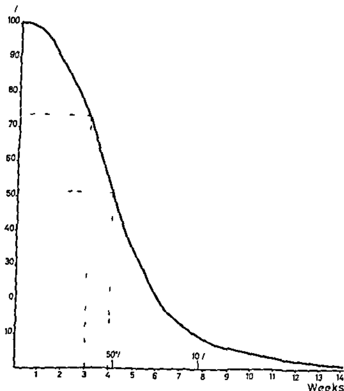


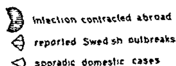
Fig 3

Length of infectious period (Calculated from 466 clinical cases of S typhi murium infection in Vaggeryd 1962 Nyström G & Broholm K A)

The frequency of Salmonella carrier state in Swedes who have not been abroad recently is not known but we have reason to believe that it is low In a large Swedish food industry with 1100 employees cultures for *Salmonella* were carried out 1–3 times every year During 1960 1967 a total of 20 000 cultures were made and only in 9 cases was Salmonella isolated Five of these had most probably contracted the infection abroad while 3 were discovered in connection with local outbreaks Only 1 carrier was foumed in whom the infection was of unknown origin (1 3)

Study 2

During 1963 the total number of reported *Salmonella* cases was 1 344 In 1 247 of these cases epidemiological information was obtained through questionnaires sent to the patients and from notification forms kindly made available by the County Officers of Public Health In 63 per cent of the cases the infection was considered to have been contracted abroad (Fig 4) This group included all patients who had been ill with gastro intestinal symptoms during their stay abroad and who were found to excrete *Salmonella* bacteria after they had returned home



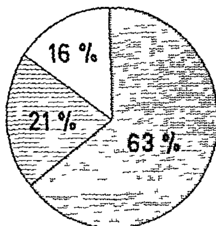


Fig. 4

Origin of Salmonella infections in Sweden 1963. 1344 bacteriologically confirmed cases

It also included patients who had not been ill but were found to excrete *Salmonella* in specimens collected within a month after their return. 21 per cent of the cases were connected with known outbreaks within Sweden while the remaining 16 per cent were sporadic cases. Some of the latter may have been secondary to imported cases although in many of them the origin was obscure. The high relative proportion of imported cases (63 per cent) was influenced by the fact that only few major outbreaks of salmonellosis occurred in Sweden in 1963. The results may also have been influenced by the search for *Salmonella* carriers described in Study 1.

Table 2 illustrates the number of Swedish tourists visiting different countries and the number of cases reported to be infected with *Salmonella* after visits to these countries. Information about the number of visitors has been obtained from the tourists agencies of the countries concerned. The table gives a rough idea of the countries in which *Salmonella* infections have been contracted. It does not permit any conclusions about the risk of contracting an infection in any particular country as this risk may be influenced by several other conditions such as the length of the sojourn, standard of accommodation, selection of food stuffs etc.

During 1963 65 different *Salmonella* sero types were isolated. Some of the sero types found in tourists were very uncommon in Sweden (Fig. 5). Thus 148 cases of *S. haifa* infection were reported in 1963. Only three of these had contracted their infection in Sweden whereas

TABLE 2

Number of Swedes Visiting Certain Foreign Countries during 1963 and the Number of Swedes Reported to be Infected with Salmonella after Visits to these Countries

Country	Number of visitors	Number of persons infected
Germany	1 128 8[illegible]0	5
Italy	371 200	[illegible]56
Spain–Mallorca–Canary Island	121 503	112
France	110 000	4
Austria	87 030	1
Switzerland	[illegible] 000	2
England	57 000	5
Netherlands	36 000	10
Jugoslavia	[illegible]7 640	12
Greece	24 748	12[illegible]
USA	13 991	0
USSR	12 770	1
Portugal	[illegible] 9[illegible]7	4
Israel	4 548	4
Roumania	3 000	59
Japan	2 000	1
Turkey	1 891	1
Hungaria	1 500	0
Other countries	number not available	193

Including travellers passing through the country by air train etc

the remaining 145 cases were infected while visiting a certain area in Italy Similarly *S abony* was isolated almost exclusively from tourists visiting Roumania

DISCUSSION

It seems obvious from this study that the increasing incidence of Salmonellosis in Sweden as well as the increasing number of sero types found during the last few years to a great extent may be due to an increasing importation of *Salmonella* This importation may increase considerably the risk of a secondary spread within Sweden by means of food borne infections This risk becomes greater and the consequence more important as food handling becomes centralized to a greater extent There is also a latent risk for family outbreaks especially in families with small children Experience has shown that children may be infected by much smaller infecting doses than adults (2)

In addition to the epidemiological risks attached to the importation of *Salmonella* the infections are a considerable nuisance to the individual traveller He runs the risk of having his vacation totally or partly spoiled and in certain cases a *Salmonella* infection also can be a serious threat to his health

The problems connected with the control of *Salmonella* infections

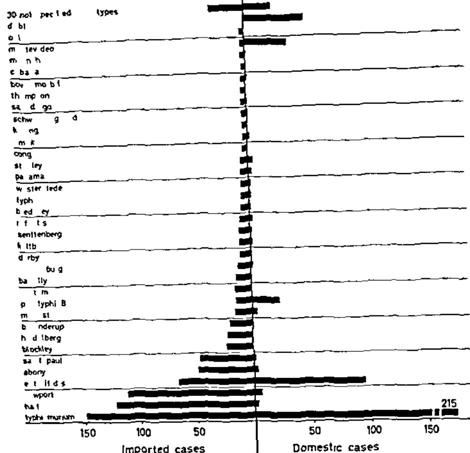


Fig 5

Imported and domestic cases of Salmonellosis in Sweden distributed according to sero types

are very difficult A possible control measure would be that every person concerned with food handling who has been abroad should leave a faecal specimen for culture on his return home Several big abattoirs and other food handling enterprises in Sweden have already started such controls on their own initiative

Since certain hotels and popular holiday resorts have been found to have endemic strains of *Salmonella* which infect the tourists in high frequency it is suggested that travelling agencies pay more attention to the hygienic conditions in their choice of destinations and hotels Such concern might influence the development towards a better food and water hygiene and safer accommodation for the tourists

The possibilities of influencing the incidence of *Salmonella* infection

by means of vaccination or drug prophylaxis will be discussed in the following paper

SUMMARY

During the last few years the number of cases of salmonellosis in Sweden has increased. Epidemiological data indicate that 63 per cent of the cases reported during 1963 had contracted their infection abroad. In some groups of travellers the mean frequency of salmonellosis was as high as 13 per cent. The risks involved in this importation are discussed.

REFERENCES

1 *Ask F* Personal communication
2 *Hook E W* *Salmonellosis. Certain factors influencing the interaction of Salmonella and the human host* Bull N Y Acad Med *37* 499–512 1961
3 *Lundbeck H* In Salmonella som problem i livsmedelshanteringen Sv inst for konservforskning rapport *191* 61–67 1966
4 *Nystrom G & Broholm K A* Vaggerydsepidemin 1962 Report to the Medical Board of Sweden 1–46 1963

Acta path microbiol scandinav 74 405–413 1968

The National Bacteriological Laboratory Stockholm Sweden

SALMONELLA INFECTION IN TOURISTS

2 *Prophylaxis against Salmonellosis*

By

L O MENTZING and O RINGERTZ

Received 24 iv 68

Part I of this series (14) showed that the increasing number of *Salmonella* infections acquired abroad constitutes an epidemiological problem It was urgent therefore to investigate the possibilities open to the travellers to protect themselves against such infections during their sojourn abroad Except for general hygienic measures two main types of prophylaxis are used at present by Swedish tourists namely TAB vaccination and the prophylactic use of halogenated oxyquinolines

The composition of the Swedish TAB vaccine is shown in Table 1

It is evident from the composition of the vaccine that it is primarily intended to protect against *S typhi* and *S paratyphi* infections i e the more serious types of *Salmonella* infection Many other sero types of *Salmonella* have antigens in common with the vaccine while others have not Little is known about the relation between the serological pattern and the protective effect According to the composition of the vaccine TAB vaccination could be expected however to protect against infection by only a limited number of Salmonella sero types

Occasionally various drugs have been used to influence the composition of the intestinal flora in order to avoid intestinal infections Due to a general temperance in the use of antibiotics in Sweden and to the risk of side effects these drugs are not used prophylactically against intestinal infections On the other hand halogenated oxyquinolines are commonly used and generally they can be obtained without a doctor s prescription Frequently these drugs are advertised in the press and recommended for prophylactic use against intestinal infections (tourist s diarrhea Canary disease summer disease etc)

Several investigations of the activity of these compounds against microorganisms *in vitro* have been performed (4 6 16) Such investigations have been made in Sweden by *Diding & Strom* in 1957 and *Thorsson* in 1962 The latter found dibromoxyquinoline antibacterially more active than diiodooxyquinoline Considerably higher concentrations of both drugs were required to inhibit growth of Salmonella than to inhibit a number of aerobic bacteria normally found in the gut

The rational grounds for the use of oxyquinolines prophylactically

TABLE 1
Composition of the TAB Vaccine

Strain of Salmonella	Proportion %	O	Antigen Vi	spec H	Non spec H
S typhi	50 0	9 12	Vi	d	–
S paratyphi B	25 0	(1) 4 (5) 12	–	b	1 2
S paratyphi A	12 5	(1) 2 12	–	a	–
S paratyphi C	12 5	6 7	Vi	c	1 5
S bovis morbificans		6 8		r	1 5
S oranienburg		6 7	–	mt	–
S newport		6 8	–	eh	1 2

are somewhat obscure partly due to incomplete information about the aetiology of many diarrheal diseases The preceeding paper of this series showed however that these diseases in tourists were often caused by *Salmonella*

Only few investigations into the prophylactic effect of oxyquinolines have been published *Hyllner & Heinlaid* (7) studied the effect of dibromoxyquinoline in tourists visiting the Canary Islands and reported a good prophylactic effect (8 8 per cent morbidity in the treated group and 60 9 per cent in the control group) This investigation includes no detailed study of the aetiology of the infections but cultures of faecal *samples obtained from some of the individuals in the control group* showing gastro intestinal symptoms indicated that they were not caused by *Salmonella* or *Shigella*

Kean and co workers (9 10 20) have studied gastro intestinal infections among travellers to Mexico They found no significant difference between the prophylactic effects of iodochloroxyquinoline and placebo Faecal specimens were taken and cultured but neither *Salmonella* nor *Shigella bacteria were found and the authors suggested that the infections* might have been of viral origin

MATERIAL AND METHODS

During the period June 6–October 20 1963 all reported cases of Salmonellosis in Sweden were contacted If the patient was found to have taken part in a conducted tour to a country outside Scandinavia all other participants in the tour were asked to leave faecal specimens This investigation included a total of 4 587 tourists 1 835 of these belonging to travelling groups in which *Salmonella* morbidity was high were chosen for an interview investigation Usually the groups consisted of 50–100 participants who travelled by chartered flight to the Mediterranean area Accomodation and the main meals were usually arranged in the same hotel

The tourists were interviewed through questionnaires and detailed information *was received about the type of accomodation where meals were taken and special excursions* as well as about TAB vaccination prophylactic use of oxyquinolines symptoms *of disease* date of onset duration of illness etc

Travellers who *did not* fill in the questionnaire as well as those who did not leave faecal specimens were excluded *from* the material Among the remaining 1 135 individuals 2 4 were *found to* excrete *Salmonella* bacteria (19 7 per cent)

The specimens were collected on an average of 3–4 weeks after the traveller returned to Sweden and 65 different sero types were isolated. No cases of S. typhi or S. paratyphi infection were found.

In order to study the effect of TAB vaccination and prophylactic use of oxyquinolines the material was divided according to the following criteria:

Vaccination	
Complete vaccination	3 injections in 1963 or 2 injections in 1963 or 1 booster in 1963
Incomplete vaccination	1 injection in 1963 or 1–3 injections in 1962 or earlier
Not vaccinated	No injections
Prophylaxis by Means of Oxyquinolines	
Regular drug prophylaxi	Regular consumption of oxyquinolines at least in the dose recommended by the manufacturer during the time before illness or at least during half part of the sojourn if no symptoms appeared.
Irregular drug prophylaxis	Insufficient dosage or consumption during less than half but more than 1/3 of the sojourn
Sporadic drug prophylaxis + Incomplete information	Drug consumption sporadically or during less than 1/3 of the sojourn Incomplete information about dosage or duration
No drug prophylaxis	No consumption of oxyquinolines

RESULTS

Table 2 illustrates the *incidence of Salmonellosis* in relation to the measures of prophylaxis. TAB vaccination did not seem to influence the incidence of Salmonellosis. However, a significantly higher incidence of Salmonella infection was found among those who had taken drug prophylaxis regularly (28.5 per cent) than among those who did not take any such prophylaxis (17.3 per cent). This trend was found among vaccinated as well as not vaccinated individuals.

Table 3 illustrates in the same way the incidence of *gastro enteritis regardless of aetiology*. No prophylactic advantage of the TAB vaccination was found but similar to the results described in Table 2 a higher incidence of illness was recorded among those who had regularly taken drug prophylaxis (40.2 per cent) than among those who did not take any such prophylaxis (31.7 per cent). This difference was significant in the whole material as well as in the group not vaccinated. Some of these cases of gastro enteritis, however, may have been of an aetiology other than Salmonella. Therefore the *incidence of symptoms of gastro enteritis in travellers from whom Salmonella was isolated* after their return to Sweden is illustrated in Table 4. Here no significant difference was found between those who had taken drug prophylaxis regularly and those

TABLE 2

Proportion of Travellers Excreting Salmonella after the Various Prophylactic Treatment Described in the Text

	Regular drug prophylaxis	Irregular drug prophylaxis	Sporadic prophylaxis + Incomplete information	No drug prophylaxis	Total
Complete vaccination	48/169 28%	19/92 21%	1/17	31/191 16%	99/469 21.1%
Incomplete vaccination	6/20 30%	2/11 18%	2/7	9/49 18%	19/87 21.8%
Not vaccinated	19/67 28%	19/104 18%	2/36	66/372 18%	106/579 18.3%
Total	73/256 28.5%	40/207 19.3%	5/60 8.3%	106/612 17.3%	224/1135 19.7%

TABLE 3

Proportion of Travellers Showing Symptoms of Gastro Enteritis Regardless of Aetiology Distributed According to Prophylactic Groups

	Regular drug prophylaxis	Irregular drug prophylaxis	Sporadic drug prophylaxis + Incomplete information	No drug prophylaxis	Total
Complete vaccination	64/169 38%	19/92 21%	0/17	62/191 32%	145/469 30.9%
Incomplete *vaccination*	7/20 *35%*	4/11 *36%*	0/7	19/49 *39%*	30/87 *34.5%*
Not vaccinated	32/67 48%	29/104 28%	0/36	113/372 30%	174/579 30.1%
Total	103/256 40.2%	52/207 25.1%	0/60	194/612 31.7%	349/1135 30.7%

the incidence of symptoms was found to be significantly lower in the completely vaccinated travellers (54.5 per cent) than in the unvaccinated (71.7 per cent). In 214 out of 224 cases (95.5 per cent) the *Salmonella* strain isolated had one or more antigens in common with the vaccine.

In Table 5 the incidence of Salmonellosis in the consumers of different types of oxyquinolines is listed. The groups are however too small to allow conclusions concerning differences between the drugs. However in all groups the incidence of Salmonellosis was higher among travellers taking regular drug prophylaxis than in the group no drug prophylaxis.

TABLE 4

Proportion of Symptomatic Cases among Travellers Excreting Salmonella, D[illegible] According to Prophylactic Groups

	Regular drug prophylaxis	Irregular drug prophylaxis	Sporadic drug prophylaxis + Incomplete information	No drug prophylaxis	T[illegible]
Complete vaccination	26/48 54%	10/19 53%	0/1	18/31 58%	[illegible]
Incomplete vaccination	4/6 7%	1/2 50%	0/2	8/9 89%	12 [illegible]
Not vaccinated	15/19 79%	12/19 63%	0/2	49/66 74%	[illegible]
Total	45/73 61 6%	23/40 57 5%	0/5	75/106 70 8%	1[illegible]

TABLE 5

Incidence of Salmonellosis in Tourists Using Chemoprophylaxis of Different D[illegible] of Oxyquinoline

Drug	Regular drug prophylaxis	Irregular drug prophylaxis	T[illegible]
Iodochloroxyquinoline	19/72 26%	19/120 16	28/[illegible] 2[illegible]
Dibromoxyquinoline	8/20 40 %	9/23 39%	17 [illegible] 4[illegible]
Chlorchinaldol	3/11 27%	4/16 25%	[illegible]
Dibromoxyquinoline + D I rombenzoyl oxyquinoline	38/143 27%	7/38 18%	[illegible]
Diiodooxyquinoline	–	0/2	
More than one drug	5/10 50 /	1/8 13	
Total	73/256 28 5%	40/207 19.3%	
No prophylaxis	–	–	

DISCUSSION

The type of material examined composed of several groups of travellers with different incidence of Salmonellosis has many statistical disadvantages that make it difficult to interpret the differences found In order to see whether the results could be due to a heterogeneity of the material each group of travellers was studied separately If for example the material included a large group of travellers with a high Salmonella morbidity and many members of the group had taken drug prophylaxis this might have influenced the results The check revealed no evidence that a heterogeneity as to the time of sampling or use of prophylaxis was responsible for the results The same trends as shown in Tables 2–5 were found in most groups included in the material

The higher incidence of Salmonellosis found in travellers who had *taken regular drug prophylaxis during their visit abroad may be due to* several factors

1 The use of drug prophylaxis may have induced a feeling of safety thus making the traveller less careful than individuals who did not take drug prophylaxis However this hypothesis is contradicted by the fact that no similar difference in incidence of Salmonellosis was found when vaccinated and not vaccinated tourists were compared although vaccination could be expected to give the same feeling of safety On the *other hand tourists taking the trouble to use prophylactic drugs might* be a more cautious type of people who also would try to avoid infections in other ways for instance by carefully selecting food and drink Epidemiological studies have repeatedly revealed that different groups of travellers visiting the same hotel but on different occasions were infected by the same sero types of *Salmonella* If the reason for this similarity of infection were a presence of *Salmonella* carriers among the kitchen staff of the hotel this indicates that the travellers probably *had limited possibilities of protecting themselves against infections by* careful choice of food

2 The faecal specimens tested for *Salmonella* micro organisms were collected on an average of 3–4 weeks after the traveller s return Sweden At this time some of those who had been infected with *Salmonella* during their visit abroad had probably already got rid of their infections Even if the different prophylactic groups were initially infected to equal degrees results such as those given in Table 2 could be expected if the individuals who had taken drug prophylaxis developed a carrier state more easily than those who had not The figures however showed the same trend in specimens collected 1 2 3 or 4 weeks after the traveller s return to Sweden As the incidence of clinical gastro enteritis analysed according to the use of drug prophylaxis (Table 3) varied in the same way as the demonstration of Salmonella it can be assumed that the higher proportion of positive cultures found in people taking drug prophylaxis was not caused by a prolonged carrier state

3 The higher rate of Salmonella positive cultures in the drug prophylaxis group might have been due to an earlier collection of the faecal specimens in this group The check of the material excluded this explanation

4 If the group Regular drug prophylaxis included a great number of clinical cases who started their medication when the first symptoms appeared results like those seen in Table 2 could be expected However this explanation was excluded by the criteria on which the group was chosen and by the fact that most travellers in this group took their drug prophylaxis during the whole period abroad

5 To evaluate the importance of the fact that 560 of the travellers from whom specimens had been collected did not return their questionnaires some calculations have been made If all the 560 travellers among whom 115 were found to be infected with Salmonella were included in the group Regular drug prophylaxis the difference between this group and the group No drug prophylaxis was still significant The same results was obtained if all the 560 travellers were included in the group No drug prophylaxis

It seems rather unlikely that any of the objections given above could explain the results obtained in this investigation The results may therefore reflect an actual increase of the risk of contracting a Salmonella infection by the regular use of drug prophylaxis with oxyquinoline derivatives

The group Sporadic drug prophylaxis + Incomplete information exhibited a low incidence of Salmonellosis and a low number of travellers with symptoms of gastro enteritis The group was however somewhat heterogenous and many of the travellers in this group had given incomplete information about their consumption of drugs Therefore they may also have given an information about their symptoms that was less complete than the information obtained from other travellers Another possible explanation is the stimulation of the intestinal micro flora by subinhibitory doses of the drugs This however requires further investigations

Miller et al (13) and *Meynell* (12) demonstrated that prophylactic treatment with streptomycin increased the susceptibility of mice about 100 000 times to a streptomycin resistant strain of *S enteritidis* The natural resistance to the infection could be restored by feeding the mice faeces suspensions or a filtrate of faeces *Miller et al* also stated that this effect was due to certain types of micro organism in the intestine Normal resistance could not be restored only by the aerobic bacteria normally found in the intestine of the animals Some strains of Bacteroides were found to produce a substance having a bacteriostatic and in high concentrations a bacteriocidic effect on Salmonella

Using the same techniques as *Meynell & Miller* we have demonstrated that oxyquinoline derivatives also significantly increase the susceptibility of mice to *Salmonella* infections (15)

The *in vitro* tests with oxyquinoline derivatives referred to earlier in this paper showed that some strains of the normal intestinal flora are inhibited at concentration levels at which most *Salmonella* strains are not. It therefore seems reasonable to believe that a mechanism of the same type as the one suggested by *Miller & Meynell* could be responsible for the effects of oxyquinolines found in the tourists.

In the *in vitro* tests carried out by us (15) 100 out of 101 different *Salmonella* strains were found to be more resistant to oxyquinolines than the majority of the aerobic bacteria normally found in the gut.

As shown in Table 2, vaccination against typhoid—paratyphoid did not change the frequency of Salmonella infections. The figures given in Table 4, however, indicate that TAB vaccination may have reduced the proportion of cases showing symptoms of such infection.

The same table illustrates that drug prophylaxis, if used by travellers who became infected with *Salmonella*, did not seem to change the frequency of cases showing symptoms. It is probable therefore that drug prophylaxis made it easier for *Salmonella* bacteria to establish themselves in the gut without affecting the frequency of symptoms once the infection was established.

In this investigation no attemps were made to study the therapeutic effect of halogenated oxyquinolines or to deal with their prophylactic effect against intestinal infections other than Salmonellosis. The results suggest, however, that even if these compounds might be valuable in connection with intestinal infections of other origin they should not be used for prophylactic purposes in cases were *Salmonella* infections could be expected.

SUMMARY

Tourists visiting subtropical holiday resorts are often exposed to *Salmonella* infections. The effect of two commonly used prophylactic measures, TAB vaccination and chemoprophylaxis with halogenated oxyquinolines, has been studied in 1 135 Swedish tourists visiting Mediterranean countries. 224 of these were found to excrete *Salmonella* bacteria. No strains of *S. typhi* or *S. paratyphi* were isolated. TAB vaccination was not found to have any effect on the incidence of Salmonellosis but it seemed to reduce the number of cases showing clinical symptoms. Travellers who regularly used oxyquinolines prophylactically showed a significantly higher incidence of *Salmonella* infections (28.5 per cent) than whose who did not take drug prophylaxis (17.3 per cent). The possibilities that this increase might be due to a disturbance of the normal intestinal microflora is discussed.

REFERENCES

1. *Black, P. H., Kunz, L. J. & Swartz, M. N.:* Salmonellosis—a review of some unusual aspects. New Engl. J. Med. 262: 864–870, 1960.
2. *Bohnhoff, M., Drake, B. L. & Miller, C. Ph.:* Effect of streptomycin on susc...

tibility of intestinal tract to experimental Salmonella infection Proc Soc exp Biol Med *86* 132–137 1954

3 *Bowmer E J* The challenge of salmonellosis major public health problem Amer J Med Sci 247 467–501 1964

4 *Diding N & Ström S* Halogensubstituerade 8 oxikinoliners effekt mot olika mikroorganismer *in vitro* Sv farm Tidskr *18* 505–511 1957

5 *Hook E W* *Salmonellosis* Certain factors influencing the interaction of *Salmonella* and the human host Bull N Y Acad Med 37 499–512 1961

6 *Hyllner S* *H inlaid H* *C Thorsson K C* Fenilor (Dibromoxikinolin) – ett nytt tarmantiseptikum Sv Lakartidn 59 2431–2455 1962

7 *Hyllner S & Heinlaid H* Diarrhoea in tourists on Gran Canaria Acta gastro enter belg 27 249 1964

8 *Hyllner S & Heinlaid H* Turistdiarré och hygien Nord hyg Tidskr 45 1–13 1964

9 *Kean B H & Waters S* The diarrhea of travellers I Incidence in travellers returning to the United States from Mexico A M A Arch Indust Health *18* 148–150 1958

10 *Kean B H & Waters S* The diarrhea of travellers III Drug prophylaxis in Mexico New Engl J Med *261* 71–74 1959

11 *Kullander N E & Heinlaid H* Turistdiarre på Mallorca Sv Lakartidn *60* 2023 1963

12 *Meynell G G* Some factors affecting the resistance of mice to oral infection by *Salm typhi murium* Proc Roy Soc Med *48* 916–918 1955

13 *Miller C Ph* Protective action of the normal microflora against enteric infection an experimental study in the mouse Univ Mich med Bull 25 272–279 1959

14 *Ringert O & Mentzing L O* *Salmonella* infection in tourists I An epidemiological study Acta path microbiol scandinav 74 397–401 1968

15 *Ringert O & Mentzing L O* The effect of dibromoxyquinoline and iodochloroxyquinoline on Salmonellosis in mice Acta path microbiol. scandinav 74 414–420 1968

16 *Sackmann W & Kradolfer F* Antibakterielles Spektrum und Beeinflussung der Darmflora von Ratten durch Jodochloroxychinolin Phenanthrolin chinon und deren Kombination (Mexaform) Zbl Bakt 1 Abt Orig *181* 539 549 1961

17 *Sackmann W & Kradolfer F* Gezielte Beeinflussung der Darmflora mit Mexaform und Chlortetrazyklin Schweiz med Wschr *92* 50–53 1962

18 *Seeliger H P R & Werner H* Die Wirkung der Kombination von Jodchloroxychinolin und 4 7 Phenanthrolin 5 6-chinon auf die Faekalflora bei Personen mit gesundem Darm Arzneimittel Forsch 13 860–865 1963

19 *Silverstolpe L* *Plazikowski U* *Kjellander J & Vahlne G* An epidemic among infants caused by *Salmonella muenchen* J appl Bact 24 134–142 1961

20 *Varela G* *Kean B H* *Barrett E L & Keegan C J* The diarrhea of traveller II Bacteriologic studies of U S students in Mexico Am J trop Med *8* 353–357 1959

The *in vitro* tests with oxyquinoline derivatives referred to earlier in this paper showed that some strains of the normal intestinal flora are inhibited at concentration levels at which most *Salmonella* strains are not. It therefore seems reasonable to believe that a mechanism of the same type as the one suggested by *Miller & Meynell* could be responsible for the effects of oxyquinolines found in the tourists.

In the *in vitro* tests carried out by us (15) 100 out of 101 different *Salmonella* strains were found to be more resistant to oxyquinolies than the majority of the aerobic bacteria normally found in the gut.

As shown in Table 2, vaccination against typhoid—paratyphoid did not change the frequency of Salmonella infections. The figures given in Table 4, however, indicate that TAB vaccination may have reduced the proportion of cases showing symptoms of such infection.

The same table illustrates that drug prophylaxis, if used by travellers who became infected with *Salmonella* did not seem to change the frequency of cases showing symptoms. It is probable therefore that drug prophylaxis made it easier for *Salmonella* bacteria to establish themselves in the gut without affecting the frequency of symptoms once the *infection was established*.

In this investigation, no attemps were made to study the therapeutic effect of halogenated oxyquinolines or to deal with their prophylactic effect against intestinal infections other than Salmonellosis. The results suggest, however, that even if these compounds might be valuable in connection with intestinal infections of other origin they should not be used for prophylactic purposes in cases were Salmonella infections could be expected.

SUMMARY

Tourists visiting subtropical holiday resorts are often exposed to Salmonella infections. The effect of two commonly used prophylactic measures, TAB vaccination and chemoprophylaxis with halogenated oxyquinolines, has been studied in 1 135 Swedish tourists visiting Mediterranean countries. 224 of these were found to excrete *Salmonella* bacteria. No strains of *S. typhi* or *S. paratyphi* were isolated. TAB vaccination was not found to have any effect on the incidence of Salmonellosis *but it seemed to reduce the number of cases showing* clinical symptoms. Travellers who regularly used oxyquinolines prophylactically showed a significantly higher incidence of *Salmonella* infections (28.5 per cent) than whose who did not take drug prophylaxis (17.3 per cent). The possibilities that this increase might be due to a disturbance of the normal intestinal *microflora is discussed*.

REFERENCES

1. *Black, P. H., Kunz, L. J. & Swartz, M. N.*: Salmonellosis—a review of some unusual aspects. New Engl. J. Med. 262: 864–870, 1960.
2. *Bohnhoff, M., Drake, B. L. & Miller, C. Ph.*: Effect of streptomycin on suscep

MATERIALS AND METHODS

In order to study the susceptibility of some bacterial strains to IC and DB *in vitro* the following technique was used. As both IC and DB are poorly soluble in water and most solvents suspensions were made of 400 mg of IC and DB in 20 ml of saline solution with 10 per cent Tween 80. Serial dilutions of the suspension were thoroughly mixed with agar broth at 50° C and plates containing 1.0, 0.1, 0.01, 0.005, 0.0025 and 0.001 mg/ml of IC and DB were made.

One drop from a Pasteur pipette of a 4 hour broth culture of each strain was placed on the surface of the plates. Usually 8 strains were inoculated on the same plate and two parallel tests containing the same strains were carried out. Each strain was also put on a control plate containing no oxyquinoline.

Strains of *B. proteus*, *Str. faecalis*, *B. subtilis*, *E. coli*, *Ps. pyocyanea* and *Coli forms* isolated from faeces of mice or human subjects as well as strains of other origin were used. All *Shigella* strains and most of the *Salmonella* strains were isolated from human faeces. 36 different sero types of *Salmonella* were included and when more than one strain of the same sero type was used epidemiological data were checked to make sure that the strains did not belong to the same out break.

In vivo tests were carried out according to the following scheme. In each series male albino mice weighing 18–22 g were fed orally twice daily with 0.25 ml of a suspension of oxyquinoline tablets in saline solution. A dosage corresponding to 2 × 100 mg of dibromoxyquinoline or iodochloroxyquinoline/kg body weight per day was used. At the same time a control group was given the same volume of saline. Each group contained 20–32 mice. The medication was continued for 2–4 weeks. The feeding of the mice was made randomly in a different order every day.

On the second day approximately 4 hours after they had been fed with oxyquinoline for the third time each mouse was given a suspension of approximately 5 million *S. typhi murium* (*phagetype* 8) bacteria in broth. The animals were fed randomly using the same bacterial suspension to all the animals. A control group of mice receiving oxyquinoline was given sterile broth.

The *Salmonella* strain used had been made resistant to > 500 mcg/ml of streptomycin and the strain was not inhibited *in vitro* by 1.0 mg/ml of IC or DB.

Faeces samples were collected every day from each mouse. Approximately 10 mg of faeces was diluted 10^{-1}, 10^{-3} and 10^{-5} and each dilution was spread on Endo agar containing 200 mcg/ml of streptomycin. The plates were incubated for 24 hours at 37° C after which the colonies were counted. The *Salmonella* strain used grew readily on this medium whereas the normal intestinal flora of the mice was almost completely inhibited.

In the control group of mice that did not receive *Salmonella* bacterial counts were not carried out but samples were taken every two days and cultured undiluted.

The number of animals excreting *Salmonella* was recorded every day and the approximate number of *Salmonella* bacteria per 10 mg of faeces was calculated from the bacterial counts.

The number of deaths in each group was recorded and a post mortem examination was carried out. Cultures for *Salmonella* were made from the heart and intestine of each deceased animal. The cultures were carried out on streptomycin endo agar as well as on desoxycholate citrate agar directly and after enrichment in tubes containing combined enrichment medium according to *Kauffmann* (2). The serotype and phagetype of all the recovered strains were checked to exclude infections with strain of *Salmonella* other than the strain fed to the animals.

RESULTS

The results of the *in vitro* test are given in Fig. 1. The Minimum Inhibitory Concentration (MIC) was found to be 0.1–1.0 mg/ml for most of the strains and no significant difference between the two oxyquinolines tested was found. The *B. subtilis* strains were the most sensitive, some of them being inhibited by as little as 0.0025 mg/ml while some of the *Shigella* strains were inhibited by 0.01 mg/ml (Fig. 1). 90 per cent of

Minimum Inhibitory Concentration (mg/ml)

		00025	0005	0,01	01	1,0	> 1,0	Number of trains
E coli	DB / IC							33
Coliform bact	DB / IC							27
Ps pyocyanea	DB / IC							26
B proteus	DB / IC							29
B subtilis	DB / IC							12
Str faecalis	DB / IC							30
Shigella sonnei and flexneri	DB / IC							22
S typhi	DB / IC							6
S paratyphi A and B	DB / IC							17
Other Salmonella 36 serotypes	DB / IC							101
								303

Percentage of strains inhibited

Fig 1

Sensitivity of some bacterial strains to Dibromoxyquinoline and Iodochloroxyquinoline

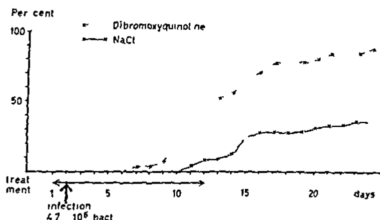


Fig 2

Death rate in mice treated with Dibromoxyquinoline

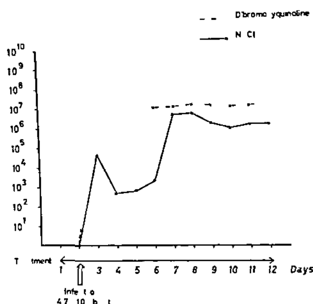


Fig 3
Mean number of Salmonella bacteria per 10 mg of faeces excreted by infected animals

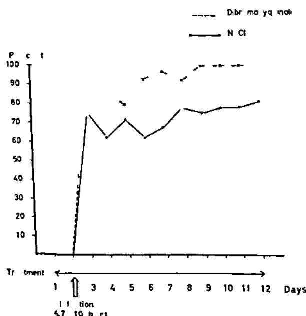


Fig 4
Per cent animals excreting Salmonella

the strains of *Enterococci* and approximately half the number of *B proteus* strains were inhibited by 0 1 mg/ml *Ps pyocyanea* and coliform bacteria were somewhat more resistant to oxyquinolines many of them being unaffected by 1 0 mg/ml All 6 *S typhi* strains tested were inhibited by 1 0 mg/ml while most strains of *S paratyphi A* and *S paratyphi B* were unaffected at this concentration 99 per cent and 90 per cent of the 101 strains of 36 different sero types of *Salmonella* grew readily on plates containing 1 0 mg/ml of dibromoxyquinoline and iodochloroxyquinoline respectively

Fig 2 illustrates the cumulative death rate for mice treated with dibromoxyquinoline (DB) and saline Approximately 1 week after the infecting dose of 4 7 × 10^6 S *typhi murium* bacteria was given the first deaths occurred A significantly higher death rate was found in the mice treated with DB than in the saline group

As shown in Fig 3 the average number of *Salmonella* bacteria/10 mg of faeces reached high values as early as 24 hours after infection In the saline control group the number of *Salmonella* bacteria decreased by the following day After another two days the number increased rapidly although it never reached a value as high as that in the DB treated group

In the DB treated group the mean number of *Salmonella* bacteria initially rose to a higher level than in the saline group and thereafter remained at this high level showing only a slight reduction during the two days following the infection

Also the number of animals excreting *Salmonella* bacteria differed in the two groups (Fig 4) In the DB treated group 90 per cent of the mice were found to have *Salmonella* in their faeces by the day after infection During the following week this figure slowly rose to 100 per cent In the saline group *Salmonella* was found initially in 70 per cent of the animals and the figure did not rise above 80 per cent

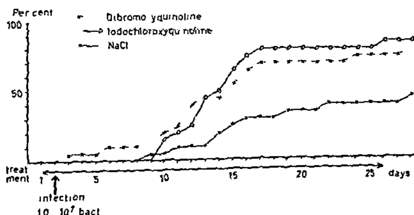


Fig 5

Death rate in mice treated with Dibromoxyquinoline and Iodochlorcxyquin line

The curves in Fig 5 illustrate the cumulative death rate in mice treated with dibromoxyquinoline iodochloroxyquinoline and saline Al most identical curves were obtained for the two oxyquinolines while the death rate was considerably lower in the saline group

DISCUSSION

The results obtained in this study conform well with those of other investigations (3 10 4) It is obvious that the majority of the *Salmonella* strains are considerably more resistant to oxyquinolines than most other aerobic bacteria found in the intestinal flora and that only very few of the *Salmonella* strains are more sensitive to these drugs than the normal intestinal flora There is reason to believe therefore that at certain concentration levels oxyquinolines may cause changes in the intestinal microflora without affecting the *Salmonella* and thus facilitate the growth of these bacteria

The results obtained in the *in vivo* tests suggest that treatment with oxyquinolines increase the susceptibility of mice to *S typhi murium* infections The proportion of animals infected and the death rate as well as the mean number of *S typhi murium* bacteria excreted in the faeces seems to be increased by the treatment

The results were similar to those obtained by *Meynell* (5) using streptomycin Some of the findings also indicated that the mechanism causing the increased susceptibility may be similar to that described by Meynell Thus as shown in Fig 3 the marked reduction in the number of *S typhi murium* bacteria found in the faeces of normal mice on the first days after excretion of the infecting dose was not found in the animals treated with oxyquinoline As outlined by *Meynell* this result may indicate that the *S typhi murium* bacteria could start multiplying immediately after entering the gut of the treated mice

The changes of the normal microflora have not been studied in the *in vivo* tests The results obtained in the *in vitro* tests however indicate that such changes could occur and that the drugs may inhibit some strains of the aerobic microflora without affecting the *S typhi murium* strain

SUMMARY

In an *in vitro* study of the antibacterial spectrum of dibromoxyquinoline and iodochloroxyquinoline over 90 per cent of the *Salmonella* strains were found to be more resistant to these drugs than most other aerobic bacteria commonly found in the intestines Tested *in vivo* the drugs were found to increase the susceptibility of mice to *S typhi murium* infections increasing the death rate as well as the proportion of animals infected and the mean number of *Salmonella* bacteria excreted

REFERENCES

1 *Black P H Kun- I J & Swart M N* Salmonellosis—a review of some unusual aspects New Engl J Med 262 864–870 1960

2 *Boecker E & Kauffmann F* In Bakteriologische Diagnostik by J Springer Berlin 1931 p 21–23

3 *Diding N & Strom S* Halogensubstituterade 8 oxikinoliners effekt mot olika mikroorganismer *in vitro* Svensk farm tidskr 61 505–511 1957

4 *Hylliner S Heinlaid H & Thorsson K G* Fenilor (Dibromoxikinolin) – ett nytt tarmantiseptikum Sv Lakartidning 59 2131–2435 1962

5 *Meynell G G* Some factors affecting resistance of mice to oral infection by Salm typhi murium Proc Roy Soc Med 48 916–918 1955

6 *Miller C Ph* Protective action of the normal microflora against enteric infection an experimental study in the mouse Univ Mich med Bull 25 272–279 1959

7 *Miller C Ph Bohnhoff M & Rifkind D* Effect of an antibiotic on the susceptibility of the mouse s intestinal tract to Salmonella infection. Trans Am clin climat Assoc 68 51–58 1956

8 *Ringert O & Ment ing L O* *Salmonella* infection in tourists 1 An epidemiological study Acta path microbiol scandinav 74 397–404 1968

9 *Ringert O & Ment ing I O* *Salmonella* infection in tourists 2 Prophylaxis against Salmonellosis Acta path microbiol scandinav 74 405–413 1968

10 *Sa kmann W & Kradolfer F* Antibakterielles Spektrum und Beeinflussung der Darmflora von Ratten durch Jodochloroxychinolin Phenanthrolinchinon und deren Kombination (Mexaform) Zbl Bakt 1 Abt Orig 181 539–550 1961

Acta path microbiol scandinav 74 421-430 1968

Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt University of Oslo
Oslo Norway Head Professor dr med S D Henriksen

A NEW SPECIES, *SPHAEROPHORUS INTERMEDIUS*, ISOLATED FROM EMPYEMA

By

TOM BERGAN and BERIT HOVIG

Received 25 iv 68

The purpose of this paper is to report a case of an infection due to an anaerobic non spore forming Gram negative rod As far as the authors are aware no description of a species with the same characteristics as our strain has been reported previously

L H 63 year old male The patient had suffered from angina pectoris since 1951 and he had attacks of cardial infarction in 1952 and 1955 He had gradually increasing dyspnoea during the last few years

From June 1967 the patient had coughing and haemoptysis In August there f re an X ray examination of the lungs was performed This revealed a tumour like infiltration in his left upper hilar region Subsequently he was admitted to hospital on August 22 1967

On admission the patient s general condition was good SR was estimated to 45 mm/hour and leucocyte count (l c) 9 400 Planigraphic X ray examination demonstrated a cavity filled with fluid within the tumour mass Consequently the patient was tentatively treated with ampicillin for eighteen days This did not alter the process as judged by the results of X ray and blood examinations

A left sided pneumonectomy was performed on September 13 The tumour was localized to the central part of the upper lobe constricting the main bronchus and infiltrating the pleura Histological examination showed a poorly differentiated carcinoma The resection borders and the peribronchial lymph nodes were devoid of tumour cells

Postoperatively the patient had a slight rise in temperature During the first week he received penicillin and streptomycin the second week, his treatment was changed to chloramphenicol After two weeks he was subfebrile and the antibiotic was withdrawn On September 15 the SR was estimated to 78 mm/hour and l c was 16 900 on September 28 the SR was 93 mm/hour and l c was 12 800

During the third postoperative week the patient gradually ran elevated temperatures again and on October 6 a pleural puncture was performed The air pressure in the pleural cavity was slightly raised but a bronchopulmonary fistula could not be demonstrated Two ml of a blood stained cloudy fluid was removed and sent for bacteriological examination From October 7 ampicillin was tried for four days without reducing his temperature then the medication was altered to doxycyclin and some temperature decrease occurred However the patient acquired a copious haemorrhagic purulent sputum and a bronchopleural fistula appeared on October 10 Sixteen days later a pleurocutaneous fistula to the operation wound developed

Reexaminations by X rays during the postoperative period showed various degrees of a left sided pleural exudate and a few right sided bronchopneumonic infiltrations (Fig 1) During the same period ten cultures from sputum and the larynx for *Mycobacterium tuberculosis* were negative

The patient was discharged to another hospital on October 27 while still being



Fig 1

Chest roentgenogram taken October 25th 1967 Bronchopneumonialike infiltrations in the right lower lung field Exudate in the left pleural cavity

treated with doxycyclin The treatment was continued till January 1968 His fistulas closed during November 1967

The patient died on February 14 1969 An autopsy was not performed

MATERIALS AND METHODS

Bacteriological specimens The fluid sample on October 6 was subject to common bacteriological examinations including an anaerobic culture in thioglycollate broth

Blood specimen Blood was sampled on November 6 1967 for agglutination tests

The media used are listed in the Tables 1–3 Standard media based on Difco or Oxoid preparations have been used All test tube cultures were anaerobized by the pyrogallic acid method (12)

According to *Suzuki et al* (16) growth inhibition by dyes was done with a 1 100 000 w/v concentration of Nile blue brilliant green gentian violet Victoria blue and crystal violet in a slightly changed basal medium of the following composition Glucose 2 g Nutrient Broth (Difco) 16 g Yeast Extract (Difco) 5 g NaCl 1 g l ascorbic acid 0 1 g l cystin 0 1 g Agar No 3 (Oxoid) 7 g distilled water 1 000 ml The ingredients were dissolved the pH adjusted to 7 0 the dyes added and the medium dispensed on tubes for autoclaving 15 minutes at 121 C

RESULTS

Microscopy The strain is Gram negative After growth on artificial media relatively uniform rods appeared (0 4–0 5 μ × 1–3 μ) (Fig 2) No spores were identified

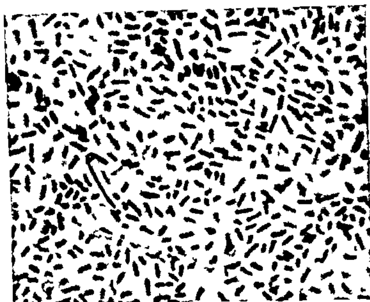
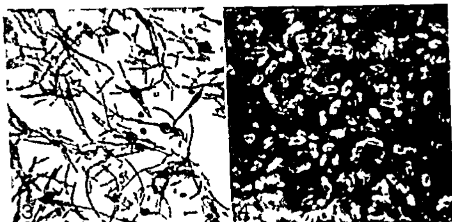


Fig 2

Rod forms from a 3 days old growth on human blood agar Gram stain × 2700



Figs 3–4

Fig 3 Sphaeroid bodies from over night primary culture in thioglycollate broth Gram stain × 2000

Fig 4 Capsules demonstrated by *Moller* stain (13) from 4 days old growth on human blood agar × 2700

Direct microscopy of the pleural exudate demonstrated marked pleomorphism of the Gram negative rods with sphaeroid elements and ballooned spindle shaped cells A similar picture was observed from the primary thioglycollate broth culture although the sphaeroid bodies were somewhat less numerous (Fig 3) The oval bodies were located



Fig 5
Colonies on human blood agar after 4 days growth in an anaerobic jar with H atmosphere × 10

subterminally and centrally They were 1 4–4 2 μ wide with a medium size of 2 8 × 2 8 μ The primary culture also showed some filiform elements In all subsequent subcultures only a very rare ballooning was seen No increase was seen in old cultures or after growth at 40 C *Sphaeroid bodies were induced by penicillin and ampicillin A Neisser* stain revealed metachromatic inclusions in some rods whereas others were evenly stained

By the staining methods of *Butt et al* (4) and of *Moller* (13) a narrow capsule was observed (Fig 4) By the former method the capsules were of widely different thickness on the average 0 2 μ

Colonies The colonies were mucoid and partly amoeboid (Fig 5) 0 5–1 mm after two days growth on blood agar sharply demarcated with entire edges and convex shiny smooth surfaces The centres were more grayish the peripheries transparent They had a soft butyrous consistency

Odor Blood agar cultures had a faint uncharacteristic sour smell after three days

Haemolytic activity No change of blood agar was observed after three days However after four days a haemolytic digestion appeared on human agar but not on cow blood agar This appeared as zones of clearing resembling beta haemolysis and extended about 1 mm from the border of the colonies

Motility The strain was not motile as evidenced by hanging drop *and growth in semisolid agar*

Growth No growth was obtained under aerobic conditions either on solid or in liquid media The bacterium exhibited even turbidity in 1 per cent peptone water glucose broth *Todd Hewitt* broth and phosphate broth without glucose No serum or carbohydrates were needed for growth although fermentable sugars did stimulate growth The optimal temperature was 37 C in both thioglycollate broth without dextrose or indicator and *Todd Hewitt* broth The strain survived 56 C for an

hour but was killed by 70° C for 20 minutes To determine oxygen susceptibility tube cultures with glass rods were shaken aerobically at 37 C similar to the technique described by *Midtvedt* (12) For this purpose Thioglycollate Broth (Difco) was inoculated with 0 2 ml of a 2 days old culture in the same medium Subcultures in thioglycollate broth showed that the strain survived 24 but not 48 hours aeration No growth was observed on *Sabouraud* agar plates or in glucose broth with 6 5 per cent NaCl Results obtained with a series of dye containing media developed by *Suzuki et al* (16) are shown in Table 1 A medium developed by *Goldberg et al* (6) showed that 40 per cent ox bile inhibited growth whereas agar with 10 and 20 per cent stimulated growth

TABLE 1

Growth Inhibition by Dyes According to Suzuki et al (16)

Brilliant Green	+(+)	Victoria Blue	++
Crystal Violet	—	Gentian Violet	—
Nile Blue	++(+)	Basal Medium	+++

The + to +++ indicate increasing growth intensity No growth was observed in media where — is indicated

TABLE 2

Biochemical Reactions Other than Fermentation

Oxidase (*Kovacs* method (10))	—	Nitrate reduction (9)	—
Catalase	+	Gelatin liquefaction †	—
Indole (in peptone water)	—	Ox serum liquefaction	—
Citrate (*Koser* s medium (9))	—	Egg white liquefaction	—
Urease (*Christensen* s medium (9))	—	H_2S §	—
Sodium hippurate	—	Gas production	—

Tested on a glass slide with 3 per cent H_2O_2
† Nutrient broth with 15 per cent gelatin
§ Tested on the media described by *Thjötta & Jonsen* (17) on lead acetate agar and on Triple Sugar Iron Agar (Difco)
Tested under conditions of optimal growth in the media listed in (17)

Biochemical reactions The results are recorded in Tables 2 and 3 Growth was observed in all tubes with negative reactions

Litmus milk was reduced within 24 hours and coagulated after three but no digestion took place After three days the pH was 5 0 after six days 4 9 the controls had pH = 6 5 both times Skimmed milk gave similar findings

Experimental pathogenicity Injection of 0 3 ml of a two days old thioglycollate broth culture subcutaneously (s c) in the groin of a mouse and 0 5 ml s c in a guinea pig did not elicit any pathological process

Serology A test tube agglutination with 0 5 ml of the patient s s and 0 5 ml of a saline suspe

positive to a titre of 1/16 after 24 hours at 37 C A suspension of cells boiled for two hours and a half was not agglutinated Sera from two normal persons did not agglutinate either suspension

TABLE 3
Fermentation of Alcohols and Carbohydrates

Substance	Potentiometer Method pH	Substance	Potentiometer Method pH
Monosaccharides		Saccharose	5.2
Pentoses		Trisaccharide	
l Arabinose	6.3	d Raffinose	5.[illegible]
Rhamnose	6.3	*Polysaccharides*	
d Xylose	5.3	Cellobiose	6.[illegible]
Hexoses		Inulin	6.2
d Fructose	5.0	Starch	5.4
d Galactose	5.2	Alcohols	
d Glucose	5.1	Adonitol	6.3
d Mannose	5.1	Glycerol	6.2
Disaccharides		*d Sorbitol*	6.3
d Lactose	5.2	Mannitol	6.3
Maltose	5.2	Test Medium without Carbohydrate	6.3

Tested in Thioglycollate medium without Dextrose or Indicator (Difco) containing 1 per cent of the substrate sterilized by filtration Inoculated with aliquots of 0.2 ml of a 48 hour thioglycollate broth culture Uninoculated controls showed pH from 6.4 to 6.7 After 10 days the pH fell a further 0.1 to 0.3 in all tubes

Antibiotic sensitivity Using the method of *Ericsson et al* (5) except that cultivation was done in anaerobic jars with H_2 atmosphere we found growth inhibition corresponding to grade 1 highly sensitive with chloramphenicol erythromycin, fucidin lincomycin oleandomycin oxytetracyclin and sulfonamide The strain was resistant to ampicillin bacitracin cephalosporin colistin kanamycin methicillin nalidixic acid neomycin nitrofurantoin novobiocin penicillin polymyxin B and streptomycin The zones and corresponding sensitivity grades customarily used may however only be employed as indications in this instance due to the slow growth of the strain and the anaerobic test conditions The minimum inhibitory concentration (MIC) was tested in Penassay Broth (Difco) with 5–1–0 5–0 2–0 05 mcg/ml of penicillin and of ampicillin and was found to be 5 mcg/ml for each

DISCUSSION

The isolation of this strain as the sole species in a human empyema indicates that it is pathogenic to man The entry of the organism and the establishment of infection was certainly aided by the patient's carcinoma and his consequently lowered state of resistance as well as by the operation The strain was not pathogenic to healthy laboratory animals however

The virulence of some *Bacteroides* and *Sphaerophorus* species is well demonstrated in experimental animals (2 14) Many reports of fatal disease have appeared in the literature (7 11) *McKay & Sprunt* (11) in 1952 reported 35 cases from a five year interval Of these 9 died with infections due to strains of *Sphaerophorus funduliformis* (under the name *Bacteroides funduliformis*)

In 1956 *Gunn* (7) reviewed the literature on *Bacteroides* infections Due to the varying practice of nomenclature within the *Bacteroidaceae*, his survey would include cases due to both *Bacteroides* and *Sphaerophorus* The most frequent infections caused by these organisms are localized to the respiratory organs including the pleural cavity Other common categories are septicaemias (often *post partum*) and appendicitis At times there has been preceeding abdominal surgery accidental lesions or underlying debilitating disease (3) The habitats of these organisms are primarily the gastro intestinal tract the female genital organs and the respiratory mucous membranes

The family *Bacteroidaceae* is subdivided into five genera (2) The first three form a group and is characterized by i) rarely being pleomorphic and ii) by strict anaerobioses

a) *Bacteroides* (having rounded ends and occasionally being pleomorphic)
b) *Fusobacterium* (with pointed ends)
c) *Dialister* (with small cells less than 0 15 micron wide)

The second group is distinguished by highly pleomorphic rods and consists of the two genera

d) *Sphaerophorus* (strict anaerobiosis) and
e) *Streptobacillus* (facultative anaerobes)

There is considerable disagreement regarding the classification of anaerobic Gram negative rods *Prevot* (14) classifies the genus Bacteroides of *Bergey s Manual* (2) as a separate family *Ristellaceae* This is subdivided into the genera *Ristella, Capsularis* (encapsulated) and *Zuberella* (motile) His *Ristella* comprises 37 *Capsularis* 4 and *Zuberella* 11 species *Prevot* recognizes another family the *Sphaerophoraceae* which includes the genus *Sphaerophorus* with 17 species The situation is complicated by the fact that the information given in original descriptions of new species often lack necessary details

A more rational division into genera based on metabolic properties would improve the situation *Suzuki et al* (16) approached this by investigating growth inhibition by a series of dyes They concluded that certain dyes inhibited the growth of *Bacteroides* but not of *Sphaerophorus* Further investigation is needed to elaborate these findings which do not aid the classification of our strain anyway

It seems that the strong emphasis put on cell morphology in the

taxonomy of this group of anaerobes is unfortunate because pleomorphism may often depend on growth environment and may appear in a wide variety of organisms under adverse conditions In the case of *Bacteroides hypermegas* for instance only older cultures exhibited ovoid bodies (8) When the pleomorphism is only demonstrable under extreme growth conditions it may easily be missed *Steen & Thjotta* (15) thus observed clostridium formed bodies in a *B serpens* from pus of a guinea pig The taxonomic importance of pleomorphism appearing only under special circumstances is difficult to assess and its significance is appraised differently In the case of *B hypermegas* for example the authors only observed sphaeroid bodies in one strain among several with the same biotype and only in old cultures of this one strain The rule thus being regular cell morphology the authors considered it a *Bacteroides Prevot* (14) on the other hand listed the species as *Sphaerophorus hypermegas*

Thus it is likely that some species of the *Bacteroidacae* are classified under *Bacteroides* when they could perhaps equally well be classified as *Sphaerophorus* It is obvious that a separation of these two genera based on such inconstant morphological criteria is not satisfactory

We are in doubt concerning the most reasonable generic relationship of our strain Subcultivation has failed to demonstrate the same distinct sphaeroid bodies and they have not appeared regularly in old cultures *or after growth at 40 C This would tend to justify a classification of* the strain under *Bacteroides*

However cell morphology in films of the pleural exudate and the primary culture showed numerous sphaeroid bodies The patient did not receive antibiotics at the time of isolation so we do not deal merely with sphaeroplasts thus induced such a transition was indeed observed with ampicillin and penicillin concentrations of 1 mcg/ml and less Under the present state of taxonomy therefore we feel that this fact together with a) a very occasional sphaeroid formation in old cultures and b) the occurrence of spindle shaped cells under ordinary conditions and c) the difference in metachromatic inclusions from cell to cell suggest classifying it as a *Sphaerophorus* But as discussed above it may be questioned whether the distinction between *Bacteroides* and *Sphaerophorus* rests on a sound basis

We have reviewed descriptions of all species of the Bacteroides *Sphaerophorus* group and have not been able to find any corresponding to our strain None of the four *Capsularis* strains listed by *Prevot* (14) has any resemblance to this *strain and no encapsulated Sphaerophorus* has probably been seen before

The species that seems most similar to our strain is *B hypermegas* Harrison & Hansen 1963 (8) *sive S hypermegas (Harrison & Hansen* 1963) Prevot 1966 (14) This is also non motile and clots milk Its action on gelatin is unknown Our strain in distinction produces catalase ferments a widely different spectrum of carbohydrates does not

ferment any polyvalent alcohol and does not require fermentable carbohydrate for growth. Our strain is markedly smaller (0.4–0.5 μ × 1–3 μ) than *B. hypermegas* (1–2.5 μ × 5–20 μ). The temperatures of optimal growth and their temperature resistance are also dissimilar.

It is therefore possible that this strain represents a new species. If this is substantiated by future investigation the name *Sphaerophorus intermedius* is proposed. The epithet *intermedius* was chosen to stress the fact that the strain seemed intermediate between *Bacteroides* and *Sphaerophorus* according to the present criteria for a separation of these genera.

This strain has been deposited as a type strain in The National Collection of Type Cultures, London, where it has received the number 10581, and in the American Type Culture Collection, where it has been assigned the number 23745.

SUMMARY

A case of empyema caused by a strain of the *Bacteroidaceae* is reported. Its biochemical activities are recorded in Tables 2 and 3.

As far as the authors are aware, no description of a strain with the same characteristics has been reported earlier, and it is believed that its characteristics are sufficiently different from any previously recognized species to justify the creation of a new species for which the name *Sphaerophorus intermedius* is proposed.

The difficulties encountered in distinguishing between *Bacteroides* and *Sphaerophorus* by the present morphological criteria are discussed. It is suggested that additional studies of the metabolic and other characteristics of the organisms are needed to arrive at a more satisfactory classification.

REFERENCES

1. *Ament, M. E. & Gaal, S. A.:* *Bacteroides* arthritis. Amer. Dis. Child. 114: 427–428, 1967.
2. Bergey's *Manual of Determinative Bacteriology*. The Williams & Wilkins Company, Baltimore, Md. 1957, Pp. 423–434.
3. *Bornstein, D. L., Weinberg, A. N., Swartz, M. N. & Kunz, L. J.:* Anaerobic infections—review of current experience. Medicine (Balt.) 43: 207–232, 1964.
4. *Butt, E. M., Bonynge, C. W. & Joyce, R. L.:* The demonstration of capsules about hemolytic streptococci with India ink or azo blue. J. Infect. Dis. 58: 5–9, 1936.
5. *Ericsson, H., Högman, C. & Wickman, K.:* A paper disk (sic) method for determination of bacterial sensitivity to chemotherapeutic and antibiotic agents. Scand. J. Clin. Invest. 6 Suppl. 11: 23–35, 1954.
6. *Goldberg, H. S., Barnes, F. W. & Charles, A. B.:* Unusual *Bacteroides*-like organism. J. Bact. 87: 737–742, 1964.
7. *Gunn, A. A.:* *Bacteroides* septicaemia. J. Roy. Coll. Surg. (Edinburgh) 2: 41–50, 1956.
8. *Harrison, A. P. & Hansen, P. A.:* *Bacteroides hypermegas* nov. spec. Antonie van Leeuwenhoek 29: 22–28, 1963.
9. *Jessen, O.:* *Pseudomonas aeruginosa* and Other Green Fluorescent Pseudomonads. A Taxonomic Study. Munksgaard, Copenhagen 1965, Pp. 38–46.

10 *Kovacs N* Identification of *Pseudomonas pyocyanea* by the oxidase reaction Nature *178* 703 1956
11 *McKay L I & Sprunt D H* *Bacteroides* infections Ann Int. Med *36* 56–76 1952
12 *Midtvedt T* Properties of anaerobic Gram positive rods capable of 7 *a* dehydroxylating bile acids Acta path microbiol scandinav *71* 147–160 1967
13 *Moller O* A new method for staining bacterial capsules Acta path microbiol scandinav *28* 127 131 1951
14 *Prevot A* Manual for the Classification and Determination of the Anaerobic Bacteria Lea & Febiger Philadelphia 1966 Pp 91–151
15 *Steen E & Thjotta T* Studies on *Bacteroides* V Cerebral abscess caused by *Bacteroides serpens* Acta path microbiol scandinav *27* 851–864 1950
16 *Suzuki S Ushijima T & Ichinose H* Differentiation of *Bacteroides* from *Sphaerophorus* and *Fusobacterium* Jap J Microbiol *10* 193–200 1966
17 *Thjotta T & Jonsen J* Studies on *Bacteroides* III Investigation and discussion of methodes [*sic*] Acta path microbiol scandinav *26* 538–554 1949

Acta path microbiol scandinav 74 431–444 1968

The Department of Histology and the Neuropathological Laboratory Department of Pathology I University of Goteborg Sweden

ULTRASTRUCTURAL DEMONSTRATION OF LYSOSOMES IN *TOXOPLASMA GONDII*

By

HANS ARNE HANSSON and PATRICK SOURANDER

Received 9 iv 68

There are several electron microscopic studies of the protozoon *Toxoplasma gondii* These have demonstrated the occurrence of several structures characterizing sporozoa especially the conoid which consists of a cylindrical or truncated conelike structure in the pointed end of the curved parasite (*Gustafson et al* 1954 *Ludvik* 1958 *Garnham et al* 1962 *Gavin et al* 1962 *Wildfuhr* 1964 1966 *van der Zypen & Piekarski* 1967)

Several toxonema or convoluted tubules occur in close relation to the conoid and extend posteriorly into the cytoplasm Several subpellicular fibrils originate in the pointed end also and extend posteriorly beneath the sevenlayered plasma membrane or pellicle The nucleus which sometimes shows large conglomerates of chromatin has one nucleolus and is surrounded by a doublelayered membrane The cytoplasm contains a prominent Golgi complex one or a few mitochondria endoplasmic reticulum ribosomes and glycogen granules

Lysosomes defined as sack like structures surrounded by a single membrane and containing certain acid hydrolytic enzymes have not been demonstrated in protozoa (*Crimstone* 1966) However cytoplasmic dense bodies have been thought to be such and have been described (*Steinert & Novikoff* 1960 *Garnham et al* 1962 *Gavin et al* 1962 *Ohsa* 1963 *Brown et al* 1965 *Sommer & Blum* 1965 *van der Zypen & Piekarski* 1967) Several acid hydrolytic enzymes have been demonstrated in protozoa including *Toxoplasma gondii* suggesting the presence of lysosomes (*Muller* 1962 *Schneider* 1964 *Brooker & Vickerman* 1964 *Sommer & Blum* 1965 *Lund et al* 1966)

This ultrastructural study of *Toxoplasma gondii* was designed to examine the hypothesis that typical lysosomes exist in the parasite

The authors are indebted to Ass Prof *F Lycke* for kindly supplying the parasites Dr *H Kalimo* and Dr *W Bondareff* for their interest and valuable discussions Miss *Margareta Persson* and Miss *Elin Olsson* for skilful technical assistance and Mrs *Ingrid Lundberg* for typing the manuscript

This study was supported by a grant (B 68 12X–82–04) from the *Swedish Medical Research Council*

MATERIALS AND METHODS

Parasites *Toxoplasma gondii* of the RH strain was used Peritoneal exudate from Swiss albino mice infected four days earlier with the parasites was obtained from the Virological laboratory Sahlgren s hospital Goteborg

Fixation The following fixatives were used 1 per cent osmium tetroxide (*Caulfield* 1957) 40 per cent osmium tetroxide in carbon tetrachloride (*Afzelius* 1962) and 1 per cent potassium permanganate in Zetterqvist s buffered salt solution (for description see *Sjöstrand* 1967)

The parasites were fixed immediately after harvesting by dripping the peritoneal exudate into the fixation solution Infected cultures of rabbit retina (*Hansson* 1968) were fixed by immersion

For demonstration of enzyme activity the parasites were fixed in 3 per cent gluteraldehyde (Fisher Chemical Co Chicago Ill U S A biological grade) for 2 hours at 4 C, or in 4 per cent formalin solution for 2 hours at 4 C Both fixatives were buffered in 0 1 M cacodylate buffer of pH 7 4 The 50 per cent gluteraldehyde was treated with activated charcoal prior to use Only transparent uncoloured stock solutions of pH 3 to 5 were u ed The formalin solution was prepared prior to use by heating an alkaline solution of paraformaldehyde (Merck Chemical Co Darmstad W Germany) to 70 C The parasites in the peritoneal exudate were centrifuged to a pellet which was embedded in 2 per cent agar and trimmed to a small block The specimens were repeatedly rinsed in large volumes of 0 1 M cacodylate buffer with 7 5 per cent sucrose of pH 7 4 at 4 C for several hours

Incubation for Demonstration of Arylsulphatase Activity

The specimens were incubated for 10 to 30 minutes at 37 C in a solution described by *Hopsu Havu et al* (1967) 40 mg of p nitrochatechol sulphate (Sigma Chemical Co St Louis Mo U S.A) was dissolved in a solution containing 1 ml of distilled water 3 ml of 0 1 M acetate buffer of pH 5 5 and 1 ml of 5 per cent barium chloride The final pH was adjusted to 5 5 with 0 2 M acetic acid In all 40 infected retinal cultures and 14 batches of parasite containing peritoneal exudate have been used

Incubation for Demonstration of Acid Phosphatase Activity

A modified Gomori reagent has been used consisting of equal parts of 0 1 M β glycerophosphate (Sigma Merck) 0 1 M acetat buffer pH 5 0 and 0 12 per cent lead acetate After incubation for 10 to 30 minutes at 37 C the specimens were rinsed for 5 seconds in 2 per cent acetic acid for removal of nonspecific lead precipitate. In all 32 infected cultures and 6 batches of peritoneal exudate have been used

Postfixation embedding sectioning and Electron Microscopy

The specimens were postfixed in osmium tetroxide dehydrated in ethanol and propylene oxide or 2 hydroxypropyl methacrylate (Rohm and Haas CMBH Darmstadt W Germany) embedded in Epon 812 and sectioned with an LKB Ultratome III The sections were examined in a Siemens E A Elmiskop either unstained or after staining with uranyl acetate and lead citrate

With a view to test the specificity of the reactions parallel control experiments were made by excluding the substrate or the capturing ions barium or lead or by heating the specimens

Electron diffraction studies were done in order to exclude the presence of non specific dense precipitates of structures with high activity of arylsulphatase in a Siemens E A Elmiskop The diffraction patterns obtained were compared with those of 1 per cent barium sulphate which were allowed to dry on a formvar coated grid as described by *Arstila et al* (1966)

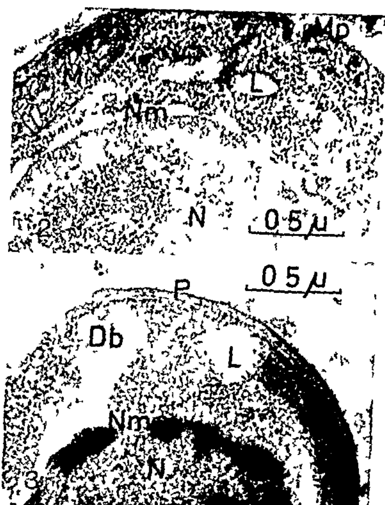
RESULTS

Vacuoles were observed in the cytoplasm of *Toxoplasma gondii* They were surrounded by a single membrane and usually two or three were observed per section often subpellicular or perinuclear (Figs 1 2 and



Fig 1

An oblique section of *Toxoplasma gondii* demonstrating the pellicle nucleus nuclear membrane endoplasmic reticulum toxonema mitochondria and Golgi complex Vacuoles bordered by a membrane and interpreted as lysosomes are seen left of the Golgi complex A dense body containing an osmiophilic myelin figure lies below the pellicle near the pointed end Fixed in cold 10 per cent osmium tetroxide in carbon tetrachloride for 30 minutes Stained with uranyl and lead



Figs 2–3

Fig 2 A micropyle is seen in the upper right corner The invagination is bordered by a membrane surrounded by osmiophilic granules forming a thick dense collar Note the club shaped internal structure of the mitochondria to the left Fixed and stained as Fig 1

Fig 3 A vacuole bordered by a single membrane is seen beneath the pellicle Note the chromatine aggregates in the nucleus Control specimen fixed in glutaraldehyde and incubated for demonstration of aryl sulphatase activity but without the substrate p nitrocatechol sulphate Postfixed in osmium tetroxide and stained with uranyl and lead

3) Osmiophilic bodies often resembling myelin figures were sometimes observed in the vacuoles (Fig 1) Their diameter usually measured 0 30 μ There was no obvious difference in the appearance size or distribution of these structures in extracellular and intracellular Toxoplasma parasites

Usually one to three dense areas bordered by a single membrane could be recognized in the cytoplasm of *Toxoplasma gondii* after in

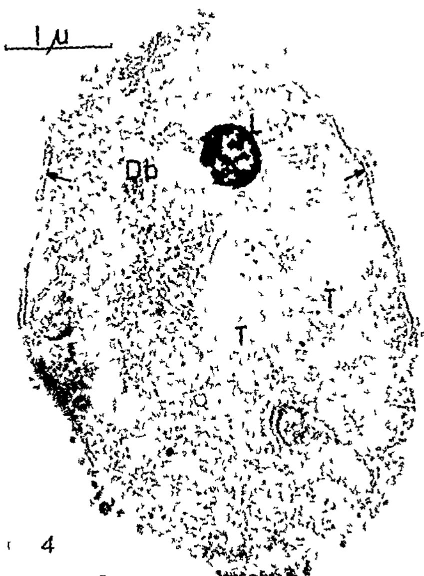
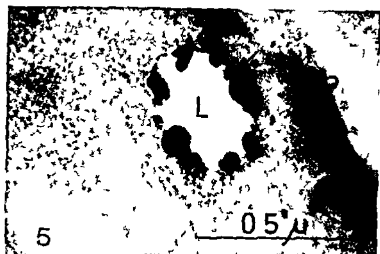


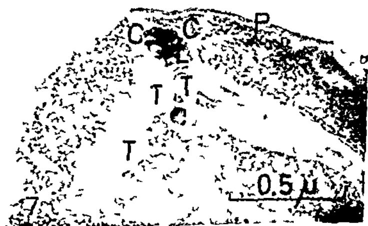
Fig 4

T x plasma parasite incubated for demonstration of arylsulphatase activity The lys me is filled with coarse dense reaction products There is an increased density in the inner unit membrane of the pellicle (arrow) Postfixed in osmium tetroxide

cubation for arylsulphatase activity All of the vacuoles described above did not show enzyme activity Those that did were readily observed because of the high contrast imparted by the barium containing reaction products The coarse precipitates were usually localized to the peripheral parts of the organelles (Figs 4 to 10) The mean diameter


L
0.5 μ
5


0.5 μ
Mp
Db
P
6


C
C
P
L
T
T
T
0.5 μ
7

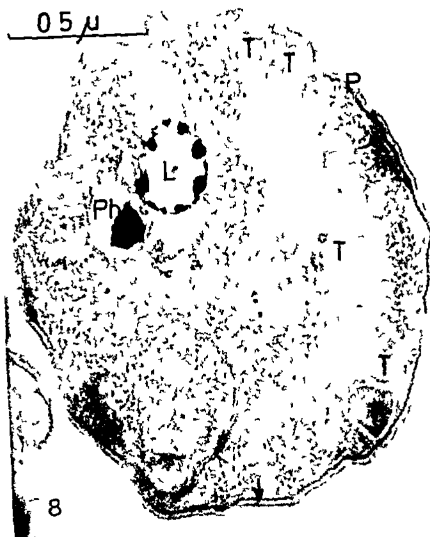


Fig 8

A lysosome with dense coarse reaction products along its membrane is seen close to but without apparent contact with a phagosome with a large irregular density Note the increased density of the pelli le (arrow) Treated as Fig 4

Figs 5–7

Fig 5 A lysosome with several dense precipitates distributed inside the distinct membrane (arrow) Incubated 10 minutes for demonstration of arylsulpha tase activity Postfixed in osmium tetroxide

Fig 6 A micropyle is seen close to a subpellicular lysosome The vacuole with some dense material to the right is tentatively interpreted as a dense body Incubated for demonstration of aryl ulphata e activity Postfixed in osmium tetroxide and stained with uranyl and lead

Fig 7 A lysosome with arylsulphatase activity is seen in close relation to the conoid and toxonema Fixed and stained as Fig 6

of the vacuoles was 0 27 μ and the range was 0 11 μ to 0 62 μ. The reacting structures described were mostly subpellicular or perinuclear. No certain relation to the conoid, toxonema, Golgi complex, endoplasmic reticulum or the subpellicular fibrils could be recognized. However, occasionally the reactive organelles were seen in close relation to the micropyle (Fig 6) or to the conoid (Fig 7). No arylsulphatase activity was demonstrated in the nucleus, mitochondria, Golgi complex, endoplasmic reticulum, conoid, subpellicular fibrils, ribosomes or micropyle. A slight activity was seen in the inner unit membrane of the pellicles and in the membrane around the two daughter parasites formed during the endodyogenetic reproduction inside the mother parasite (Fig 4 and 8).

The distribution of reaction products after incubation for demonstration of acid phosphatase activity was in agreement with that of arylsulphatase. However, precipitates were sometimes seen in the nucleus and in cytoplasmic structures (Fig 11).

The control specimens incubated without substrate and capturing ions or after heating of the specimen showed no precipitates.

Electron diffraction studies were made on structures with high arylsulphatase activity in order to evaluate the nonspecific deposition of dense material. Electron diffraction patterns were obtained from the described dense structures bordered by a membrane. They were similar to those obtained from lysosomes in mammalian cells and from the control grids with dried barium sulphate.

DISCUSSION

In the cytoplasm of *Toxoplasma gondii* of the RH strain several membrane bound cytoplasmic vacuoles, some containing osmiophilic material, were seen. They did not seem to have any consistent relation to the nucleus, mitochondria, toxonema, conoid or other cell structures. These organelles had activity both of arylsulphatase (3 1 6 1) and of acid phosphatase (3 1 3 2). The lysosomes in metazoan cells are described as round or oval, often osmiophilic organelles bordered by a single membrane with a diameter of about 1 μ. The high activities of acid phosphatase and other hydrolytic enzymes characterize the lyso

---

Figs 9–11

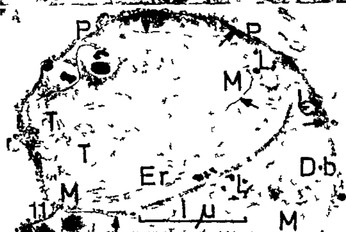
Fig 9 A lysosome and a phagosome are seen close together. Note the close c nt ct between the pellicle and the phagosome. The toxonema shows its r acteristic reticulated internal structure. Treated as Fig 6

Fig 10 The Toxoplasma parasite is surrounded by the host cell cytoplasm. Note the close relation between the lysosome and the phagosome. Treated as Fig 6

Fig 11 A Toxoplasma parasite after incubation for demonstration of acid phosphatase activity. Note the activity in the lysosomes, in the membranes surrounding the two daughter parasites in the mother parasite and in the inner unit membrane of the pellicle (arrows). There is a few coar e n n specific precipitates in the artificially widened endoplasmic reticulum. Post fixed in osmium tetroxide


0 5 μ
P
Ph
L
T
T
T
T
T
9


0.5 μ
L
Ph
P
10
T


P
P
L
M
L
T
T
D·b
Er
L
M
11
1 μ
M

somes These structures have a close relation to the phagocytic and pinocytotic activities of the metazoa cells (For review see *Wattiaux & de Duve 1966 Straus 1967*) Thus the organelles with arylsulphatase and acid phosphatase activity described in this study correspond to the lysosomes in metazoa cells The lysosomes in *Toxoplasma gondii* are of the same appearance as those in the rat kidney stained for demonstration of arylsulphatase activity with the same method as in this study (*Hopsu Havu et al* 1967) The only difference is that the lysosomes in the mammalian cells are outlined by finely granular reaction products while it is more coarse in the parasite

The two different methods for the demonstration of acid hydrolytic enzymes gave similar results as far as concerns the lysosomes However nonspecific dense precipitates were observed in the nucleus and in cytoplasmic structures after incubation for demonstration of acid phosphatase activity Therefore most of the experiments have been performed with the arylsulphatase method It was easily reproducible demonstrated activity in at least one vacuole per parasite and did not cause marked distorsion of the cellular morphology

The specificity of the reactions was tested by omitting either the substrate or the capturing ions i e lead or barium No dense material was demonstrated in the controls which corresponds to the results published for metazoan lysosomes (*Ericsson & Trump* 1964 *Wattiaux & de Duve* 1966 *Straus* 1967 *Hopsu Havu et al* 1967) The results obtained in the electron diffraction studies agree with those described by *Arstila et al* (1966) in the case of lysosomes in rat kidney stained for arylsulphatase activity *Therefore the conclusion that the histochemical* properties of the lysosomes in Toxoplasma gondii are similar to those of metazoa cells

Light microscopic histochemical studies of protozoa have demonstrated the activity of several acid hydrolytic enzymes *Muller* (1962) showed several hydrolytic enzymes in food vacuoles in *Paramecium* The activity of acid phosphatase has been observed in *Trypanosoma* (*Brooker & Vickerman* 1964) in *Euglena* (*Sommer & Blum* 1965) and in *Toxoplasma gondii* (*Lund et al* 1966) In the latter case the reaction products formed were localized to a few delimited areas in the cytoplasm Thus the results obtained in this study confirm the previous assumption by *Lund et al* (1966) that *Toxoplasma gondii* has lysosomes

Several authors described structures in *Toxoplasma gondii* and other sporozoa as lysosomes *Garnham et al* (1962) designated the toxonema as equivalent to lysosomes The vacuoles often containing osmiophilic lammellated bodies described by van der *Zypen & Piekarski* (1967) in *Toxoplasma gondii* by *Sheffield* (1966) in *Besnoitia jellisoni* and by *Sheffield & Hammond* (1966) in *Eimeria bovis* correspond in their position structure shape and size to the cytoplasmic structures described in this study The same is true for the food vacuoles in *Para*

mecium (*Schneider* 1964) and in different species of avian and primate *Plasmodium* (*Rudzinska & Trager* 1957 *Rudzinska et al* 1965 *Aikawa et al* 1966) as well as for the phagocytic vesicles in *Trypanosomes* (*Steinert & Novikoff* 1960 *Brown et al* 1965) When their pictures are compared with those in this study one observes several striking similarities It is therefore likely that the structures described in those studies are identical with lysosomes phagosomes and related organelles

Sommer & Blum (1965) described activity of acid phosphatase not only in vesicles and vacuoles but also in the Golgi complex in *Euglenia* No activity was observed in the pellicle under normal conditions However if the Euglenia cells were grown in a phosphate deficient medium and stained for demonstration of acid phosphatase activity reaction products were also seen at only one side of the notches on the outside of the pellicle The reacting parts of the notches were near tubules of the endoplasmic reticulum which occasionally showed reaction products In *Toxoplasma gondii* there does not seem to be any obvious morphological relation between the lysosomes and the toxonema mitochondria endoplasmic reticulum Golgi complex or subpellicular fibrils as previously supposed (*Garnham et al* 1962 *Lund et al* 1966) In this study none of these organelles revealed any signs of activity of arylsulphatase or acid phosphatase The pellicle showed enzyme activity both in the normal *Toxoplasma gondii* parasite and in *Euglenia* grown in phosphate deficient medium However the localizations of the reaction products were different In the latter case the precipitates were extracellular and delimited to one of the walls in the notches of the pellicle (*Sommer & Blum* 1965) On the other hand in *Toxoplasma gondii* the reaction products formed after incubation for demonstration of both arylsulphatase and acid phosphatase activity were observed only in the inner unit membrane of the pellicle (Figs 4 8 9 and 11) Precipitates have never been seen in the outer unit membrane of the pellicle or extracellularly The single unit membrane around the two daughter parasites formed inside the mother parasite during the endodyogenic reproduction showed reaction products (Fig 11) It is tempting to assume as a hypothesis on the basis of this enzyme activity pattern that the inner one of the two unit membranes of the pellicle is that originally formed around the daughter parasite The origin of the outer unit membrane of the pellicles is not known

What is the function of the lysosomes in *Toxoplasma gondii?* One possibility is that they form a part in a lysosome phagosome system in the sporozoa as in mammalian cells (as to discussion see *Wattiaux & de Duve* 1966 *Straus* 1967) *Garnham et al* (1962) demonstrated that *Toxoplasma gondii* has a micropyle i e an invagination of the pellicle into the cytoplasm (Figs 2 and 6) Their observation has been repeatedly confirmed to apply to different sporozoa (*Garnham et al* 1961 *Sheffield* 1966 *Sheffield & Hammond* 1966 *Wildfuhr* 1966 *van der Zypen & Piekarski* 1967) *Rudzinska & Trager* (1957) *Rudzinska et al*

(1965) and *Aikawa et al* (1966) described a micropyle or cytostome both in avian and primate *Plasmodium* The malaria parasite incorporates at the erythrocytic stage through the cytostome cytoplasma from its host cell into a food vacuole delimited by a single membrane The engulfed material is more or less completely digested

So far no ultrastructural cytochemical studies of acid hydrolytic enzymes in sporozoa have been published In the present study vesicles without signs of lysosomal enzyme activity have been observed (Fig 8 9 and 10) It may be assumed that *Toxoplasma gondii* and probably also other protozoa have a lysosome phagosome system similar to that observed in mammalian cells Extracellular material might be engulfed through the micropyle in a phagosome which thereafter forms a phagolysosome or digestive vacuole with a lysosome as in the metazoa cells

Another possible function of the lysosomes in *Toxoplasma gondii* is that they might be mobilized and their enzymes released into the surrounding fluid prior to the penetration of the parasite into a new host cell The cell membrane of the latter would break up and thus facilitate the penetration of the conoid on the pointed end of the parasite *Lycke et al* (1965) have demonstrated that treatment of HeLa cells with lysozyme or hyaluronidase prior to the infection with Toxoplasma parasites significantly increased the number of infected cells as compared to control cells It has been shown that these two enzymes form part of the lysosomal enzymes in at least certain mammalian cells (as to discussion see *Straus* 1967) They may possibly be present in the lysosomes of *Toxoplasma gondii* In any case it is tempting to assume that the lysosomes have a penetration facilitating effect This possibility is not excluded by the fact that there is no obvious difference between the lysosomes in intra and extracellular parasites

Additionally the lysosomes could function in the destruction of organelles in the cytoplasm of the parasite (autophagy) as described in the case of metazoa cells (for review see *Walliaux & de Duve* 1966 *Straus* 1967) Such a process probably occurs although no definite proof so far has been obtained

SUMMARY

The protozoe *Toxoplasma gondii* of the RH strain was studied with electron microscopic techniques with special regard to the occurrence of lysosomes

The parasite has one to three vacuoles delimited by a single membrane with a diameter of about 0 3 μ in its cytoplasm The vacuoles have a high activity both of arylsulphatase and of acid phosphatase The precipitates formed by the enzyme reactions are observed as dense coarse conglomerates in the peripheral parts of these structures The lysosomes in *Toxoplasma gondii* correspond to those in metazoa cells

The lysosomes in intra and extracellular Toxoplasma parasites do not differ in their number size or distribution The lysosomes have no appearent relation to the conoid toxonema subpellicular fibrils endoplasmic reticulum Golgi complex or other cell structures

The relation between the lysosomes the phagosomes and the micropyles is discussed as well as the possible penetration promoting effect of the former structures

The inner unit membrane of the pellicle shows activity both of arylsulphatase and of acid phosphatase as does the membrane around the daughter parasites formed during the internal reproduction in the mother parasite The origin of the pellicle is discussed and related to this observation

ABBREVIATIONS

C = Conoid. N = Nucleus Nm = Nuclear membrane T = Toxonema M = Mitochondria Gc = Golgi complex Er = Endoplasmic reticulum Mp = Micropyle L = Lysosome Ph = Phagosome Db = Dense body

REFERENCES

Afzelius B A Chemical fixatives for electron microscopy In Interprenation of ultrastructure edit by Harris R J C p 1–19 New York and London Academic Press 1962

Aikawa M Huff C C & Sprin H Comparative feeding mechanisms of avian and primate malarial parasites Milit Med *131* 959–983 1966

Arstila A U Jaakkola S Kalimo H Helminen H & Hopsu Havu V K Electron diffraction as the control of enzyme histochemical reactions at the ultra structural level J Microsc 5 777–790 1966

Brooker B E & Vickerman K Acid phosphatase in trypanosomes Trans Roy Soc Trop Med Hyg *58* 293–294 1964

Brown K N Armstrong J A & Valentine R C The ingestion of protein molecules by blood form of *Trypanosoma rhodesiense* Exp Cell Res *39* 129–135 1965

Caulfield J B Effects of varying the vehicle of OsO_4 in tissue fixation J Biophys Biochim Cytol 3 827–830 1957

Ericsson J L E & Trump B F Observations on the application to electron microscopy of the lead phosphate technique for the demonstration of acid phosphatase Histochemie 4 470–487 1965

Gavin M A Wanko T & Jacobs L Electron microscope studies of reproducing and interkinetic *Toxoplasma* J Protozool 9 222–234 1962

Garnham P C C Baker J R & Bird R G Fine structure of cystic form of *Toxoplasma gondii* Brit Med J *1* 83–84 1962

Garnham P C C Bird R G Baker J R & Bray R S Electron microscope studies of mobile stages of malaric parasites II The fine structure of the sporozoite of *Laverania* (= Plasmodium) *falcipara* Trans Roy Soc Trop Med Hyg 55 98–102 1961

Grimstone A V Structure and function in protozoa Ann Rev Microbiol *20* 131–150 1966

Gustafson P V Agar H D & Cramer D An electron microscope study of *Toxoplasma* Am J Trop Med Hyg 3 1008–1021 1954

Hansson H A The distribution of oxidative enzymes in retinal cultures To be published 19 8

Hopsu Havu V K Arstila A U Helminen H J Kalimo H O & Glenner G G Improvements in the method for the electron microscopic localization of arylsulphatase activity Histochemie 8 54–64 1967

Kay D Techniques for electron microscopy 2nd ed Oxford Blackwell Scientific Publ 1965

Ludvik J Morphology of *Toxoplasma gondii* in electron microscope Acta Soc Zool Bohemoslov., 22 130–136 1958

(1965) and *Aikawa et al* (1966) described a micropyle or cytostome both in avian and primate *Plasmodium* The malaria parasite incor porates at the erythrocytic stage through the cytostome cytoplasma from its host cell into a food vacuole delimited by a single membrane The engulfed material is more or less completely digested

So far no ultrastructural cytochemical studies of acid hydrolytic enzymes in sporozoa have been published In the present study vesicles without signs of lysosomal enzyme activity have been observed (Fig 8 9 and 10) It may be assumed that *Toxoplasma gondii* and probably also other protozoa have a lysosome phagosome system similar to that observed in mammalian cells Extracellular material might be engulfed through the micropyle in a phagosome which thereafter forms a *phagolysosome or digestive vacuole with a lysosome as in the metazoa* cells

Another possible function of the lysosomes in *Toxoplasma gondii* is that they might be mobilized and their enzymes released into the sur rounding fluid prior to the penetration of the parasite into a new host cell The cell membrane of the latter would break up and thus facilitate the penetration of the conoid on the pointed end of the parasite *Lycke et al* (1965) have demonstrated that treatment of HeLa cells with lysozyme or hyaluronidase prior to the infection with Toxoplasma parasites significantly increased the number of infected cells as com pared to control cells It has been shown that these two enzymes form part of the lysosomal enzymes in at least certain mammalian cells (as to discussion see *Straus* 1967) They may possibly be present in the *lysosomes of Toxoplasma gondii In any case it is tempting to assume* that the lysosomes have a penetration facilitating effect This possibility is not excluded by the fact that there is no obvious difference between the lysosomes in intra and extracellular parasites

Additionally the lysosomes could function in the destruction of or ganelles in the cytoplasm of the parasite (autophagy) as described in the case of metazoa cells (for review see *Wattiaux & de Duve* 1966 *Straus* 1967) Such a process probably occurs although no *definite* proof so far has been obtained

SUMMARY

The protozoe *Toxoplasma gondii* of the RH strain was studied with electron microscopic techniques with special regard to the occurrence of lysosomes

The parasite has one to three vacuoles delimited by a single mem brane with a diameter of about 0 3 μ in its cytoplasm The vacuoles have a high activity both of arylsulphatase *and of acid phosphatase* The precipitates formed by the enzyme reactions are observed as dense coarse conglomerates in the peripheral parts of these structures The lysosomes in *Toxoplasma gondii* correspond to those in metazoa cells

Acta path microbiol scandinav 74 445–457 1968

The Institute of General Pathology and the Department of Neurology
Aarhus Kommunehospital University of Aarhus Denmark

THE OCCURRENCE OF VIRUS AND INTERFERON IN THE SPLEEN, SERUM AND BRAIN IN MICE AFTER EXPERIMENTAL INFECTION WITH WEST NILE VIRUS

By

SVEN HAAHR

Received 8 v 68

Interferon production in experimental virus infections has repeatedly been demonstrated *e g* in the skin of rabbits in vaccinia infection (18) in the lungs of mice infected with influenza virus (16) and in the brain of mice after intracerebral inoculation with arbovirus (7 15) Circulating interferon was first demonstrated in 1963 by Baron and Buckler after intravenous inoculation of large doses of virus (1) and later after intraperitoneal inoculation of various viruses which led to viraemia (2)

In man interferon in relation to virus infection has been demonstrated in pharyngeal washings (11) in serum (23) in extract from vaccinia skin crusts (22) and in cerebrospinal fluid (12)

In most studies the interferon levels have been determined only in a few organs In some of the animal experiments interferon was induced by very large doses of virus or by viruses which do not multiply in the host for which reason the conditions concerning the quantity of interferon and its mechanism of production are scarcely comparable to those occurring during natural infections

In the study of experimental infection in mice with a neurotropic arbovirus reported below an attempt was made to simulate the course of a natural infection as closely as possible by varying the infective dose

In order to evaluate the importance of the route of infection the sensitivity to the virus was tested and the course of infection was followed on subcutaneous intravenous and intraperitoneal inoculation Virus and interferon titres were determined in the serum spleen and brain under the various experimental conditions

This study was supported by grants from *Århus Universitets Forskningsfond* and *F L Smidth & Co A/S s Jubilæumsfond*

In addition the titres of virus and interferon were compared in splenectomized and non splenectomized mice for the purpose of analysing the possible role of the spleen in the course of infection

MATERIAL AND METHODS

Viruses —West Nile virus strain Egypt 101 and Semliki forest disease virus (SFD) strain Smithburn were originally obtained from Casals in 1951 A description of passages and preparation of virus stock was given in a previous paper (13)

Media —Eagle s Basal Medium (Difco) was used for all cell cultures supplemented with serum and antibiotics as previously described (13)

Tissue cultures —Mouse embryonic cells (MF) were prepared from 18 20 day old embryos from albino mice The embryos were removed under ether anaesthesia and after removal of the head tail legs and guts the remaining tissues were minced by knives and treated with 0 01 per cent trypsin solution (Trypure Novo) under agitation by a magnetic stirrer for four or five periods of 10 minutes each The cell suspensions were pooled and after centrifugation the supernatant was decanted and the cells resuspended in the growth medium with 4 × 10[7] cells in 150 ml medium and grown in 1 litre Roux flasks at 36 C

Experimental infection —Male and female albino mice of a non inbred strain weighing 16–19 g were used in all the experiments

Intracerebral injection was given into the parietal region a little to the right of the midline under ether anaesthesia intraperitoneal injection into the umbilical region and subcutaneous injection at the tail root Intravenous injection was given into a tail vein after the mice had been placed in a thermostat at 38–40 C for 5–10 minutes Virus suspended in phosphate buffered saline (PBS) was injected in amounts of 0 05 ml

Splenectomy —After depilation and cleaning with alcohol a transversal incision 1 cm in length was made through the skin muscle and peritoneum over the spleen under ether anesthesia Following removal of the spleen and ligation of the vessels the incision was closed by suture of fascial muscle and skin in layers The same technique was used in sham operation in which the spleen was brought to the surface of the abdomen and then replaced in the peritoneal cavity Experimental infection was performed 4–5 days after the operation at which time all the animals were in a good condition

Removal of material —After the vascular plexus had been cut open under light ether anaesthesia as much blood as possible was withdrawn from the right axilla by means of a syringe The spleen brain and in some cases the liver were then removed under strictly aseptic precautions a new set of instruments being used for each organ Blood and the individual organs were pooled separately from each group usually consisting of 10 or occasionally of 8 animals

Treatment of material —After standing at room temperature the clot of the pooled blood was detached and centrifuged at 3000 r p m for 10 minutes The serum was then pipetted off and 0 5 ml of it stored at —70 C for virus titration The remainder was diluted 1 10 and used for interferon assay Immediately after the removal the pooled organs were stored at —70 C A 10 per cent suspension was made direct from the frozen organs by grinding them with sand in PBS After centrifugation at 6000 r p m for 30 minutes the supernatant was withdrawn and 0 5 ml placed at —70 C for subsequent virus titration the remainder was used for interferon assay Diluted serum and organ extract intended for interferon assay were stored at —20 C

Virus titration —The virus content in the serum and organs was determined by intracerebral inoculation into 2–4 day old mice of the material in 10 fold serial dilutions in PBS The virus titre was expressed as the exponent of the logarithmic ($\log_{10}$) dilution per 10 mg of organ or serum which caused death in 50 per cent of the animals calculated by the method of Karber

Interferon assay —Serum dilutions and organ extracts were dialyzed against Sorensen s buffer pH 2 0 and after 48 hours at +4 C dialyzed back to pH 7 4 After centrifugation at 3000 r p m for 30 minutes the supernatant was used for the assay

Determination of the antiviral activity was performed by the plaque inhibition method in secondary cultures of ME cells The cultures were grown in 50 mm

plastic Petri dishes (Nunc Roskilde) with 0 75 × 10⁶ cells in 5 ml of growth medium Incubation was carried out at 37 C in a 5 per cent CO atmosphere throughout the experiment On growth to confluence usually after 48 hours the medium was decanted and replaced by 2 ml of 2 or 4 fold serial dilutions of serum and organ extract beginning at 1 4 in maintenance medium Each dilution was tested in two cultures After absorption for 20–24 hours the cultures were challenged with 50–100 PFU of SFD virus The cultures were drained after a 1 hour absorption period and overlaid with 0 6 per cent agar (Difco) in Eagle s maintenance medium Neutral red was added 40 hours later and after a few hours the virus plaques were counted Interferon titres expressed as units per 200 mg of serum or organ were recorded as the reciprocal of the highest dilution which reduced by 50 per cent the number of plaques counted in the controls i e 50 per cent Plaque depressing dose (PDD_{50}/200 mg) A stock reference preparation of interferon was used at known titre on each assay to detect any changes in sensitivity of the system

Control interferon—This was prepared from brains of mice infected intracerebrally with West Nile virus After the development of encephalitic symptoms the mice were bled and the brains removed under aspetic precautions Otherwise the method of preparation was as stated above

Storage—The interferon was stored at —20 C or +4 C The titres of interferon stored at +4 C remained unchanged for at least 12 months

Characteristics of viral inhibitor—The viral inhibitor found in the serum dilutions and organ extracts exhibited the following characteristics

a Stable at pH 2 for 48 hours
b Not sedimented at 105 000 G for 2 hours
c Inactivated by trypsin 0 5 mg/ml for 1 hour at 37 C
d Not inactivated by RNase 0 5 mg/ml for 1 hour at 37 C
e Partly inactivated at 56 C for 30 minutes
f Did not reduce the titre of SFD virus preparations upon direct contact in a cell free medium for 1 hour at 37 C
g Did not protect human fibroblasts from virus challenge

These properties are in keeping with mouse interferon described by other investigators (6)

No mortality was found after intracerebral injection into suckling mice of dialyzed organ extracts or serum dilutions Neither was any decrease of interferon titres found after treatment with anti West Nile virus serum indicating that all of the infectivity and interfering property of West Nile virus was inactivated by dialyses at low pH

RESULTS

Variation in Infective Dose

Experiments with variation in the infective dose given intraperitoneally were repeatedly performed in several groups of mice The course of the resultant infections on administration of the various doses was in all experiments fundamentally as shown in Figs 1–3

The course of infection is shown for three dosage levels of virus viz one killing all infected animals (Fig 1) another causing a high mortality among the animals (Fig 2) and a third which produced disease in some animals but which led to death only in exceptional cases (Fig 3) Distinct viraemia was revealed after all three dosages increasing in severity from the first to the fourth day at which time the peaks for all three dosages occurred with only a slight difference in the levels The curve shows a steeper increase after the largest doses while the post maximal decreases are roughly identical after the three dosages

The amount of circulating interferon showed a greater dependance on the dose i e it increased with an increasing dose level In the ex-

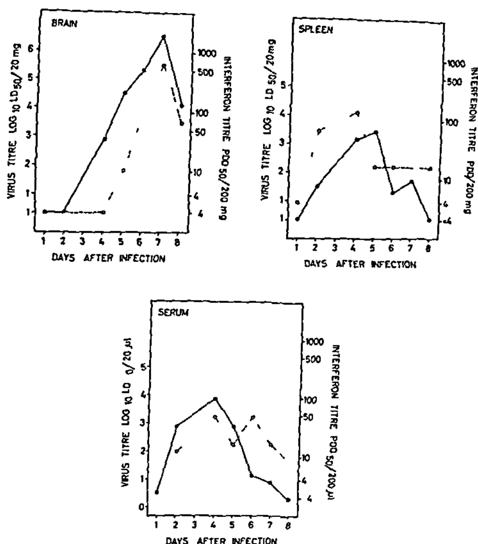


Fig 1

Titres of virus (●——●) and interferon (○——○) in the brain spleen and serum of 16–19 g mice inoculated intraperitoneally with 500 intracerebral suckling mice LD_{50} of West Nile virus causing death of 100 per cent of the infected mice

periments plotted in the diagrams the interferon level was unfortunately not determined on the first day for the largest infective dose but in other experiments with the same infective dose and route of infection no interferon could be traced in the serum on the first day On all three dosages the highest interferon levels were seen from the fourth to the sixth day

Irrespective of the infective dose no virus was present in the spleen on the first day but after that time the animals infected with the largest dose showed increasing titres reaching a peak on the fifth day On administration of the smallest dose virus could not be demonstrated

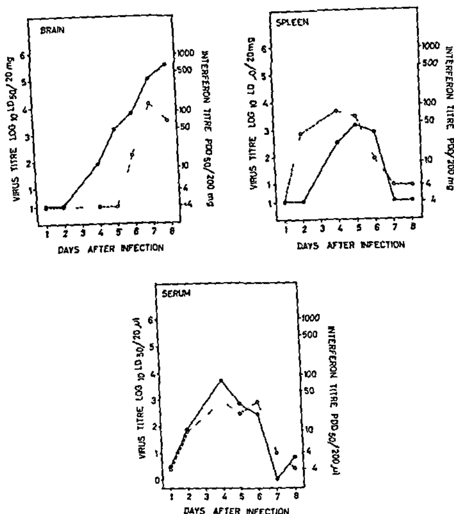


Fig 2

Titres of virus (●——●) and interferon (○——○) in the brain spleen and serum of 16–19 g mice inoculated intraperitoneally with 6 intracerebral suckling mice LD_{50} of West Nile virus causing death of 90 per cent of the infected mice

until on the fourth day but in other experiments with the lowest dose it was in a few cases impossible to reveal virus in the spleen in spite of viraemia In all experiments it was possible to demonstrate interferon in the spleen with a distinct correlation between the size of the infective dose and the amount of interferon Interferon was invariably present in the spleen at an early juncture in the course of infection often before it was possible to demonstrate virus in the organ

In some of the experiments the liver was studied for virus and interferon At the lowest dose level neither virus nor interferon could be revealed With higher doses virus could not be demonstrated during

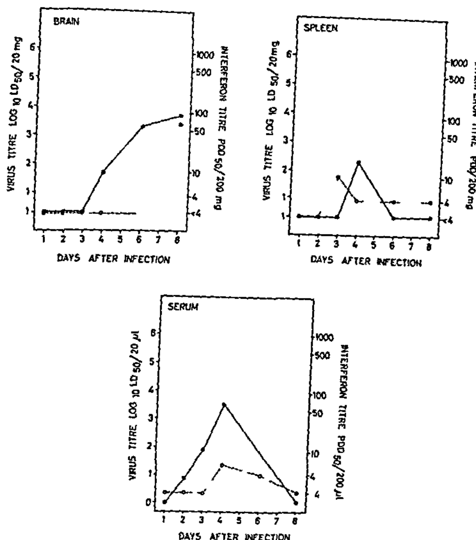


Fig 3

Titres of virus (●——●) and interferon (○——○) in the brain spleen and serum of 16–19 g mice inoculated intraperitoneally with 3 intracerebral suckling mice LD_{50} of West Nile virus causing death of 10 per cent of the infected mice

the first few days but later low virus titres were occasionally obtained ($LD_{50} \leqq 10^{1.7}$) Similarly interferon could not be traced in the liver during the first few days and only low titres ($\leqq$ 10 per 200 mg of organ) were obtained on the fourth to the seventh day

In the brain *i e* the target organ no virus could be demonstrated during the first two or three days The subsequent increase in the virus titre was steepest after highest infective dose while the lowest dose resulted in a flattening of the curve on the sixth to the eight day after infection with a relatively low maximum titre In supplementary ex periments the individual animals within the same group showed virus

titres for the brain of a fairly uniform level After all three dosage levels interferon in the brain could not be demonstrated until the fifth to the eighth day after the inoculation i e several days later than the incipient increase in the virus titre In these experiments the most distinct interferon production was also seen after the high infective doses

Variation in the Route of Infection

The sensitivity of the experimental animals to the neurotropic West Nile virus was studied by virus titration after inoculation by the sub cutaneous intravenous intraperitoneal and intracerebral routes From Table 1 it appears that while the sensitivity was unchanged after intra peritoneal intravenous and subcutaneous inoculation it was about 4 times as high after inoculation by the intracerebral route

TABLE 1

Fifty Percentage Lethal End Point of the West Nile Virus in Two Different Titrations in 16–19 G Mice Inoculated by Various Routes

Route of inoculation	Titration 1 LD_{50}	Titration 2 LD_{50}
Intraperitoneal	$10^{-3.6}$	$10^{-4.2}$
Intravenous	$10^{-3.7}$	$10^{-4.3}$
Subcutaneous	$10^{-3.6}$	$10^{-4.2}$
Intracerebral	$10^{-4.2}$	$10^{-4.8}$

A comparison of the course of the infection and of the interferon production after administration of the virus by different routes in several series of experiments with different infective doses showed that the course after subcutaneous and intravenous inoculation was essentially the same as described for intraperitoneal inoculation

On the other hand intracerebral inoculation resulted in a widely different course Fig 4 shows the conditions on inoculation with a lethal dose of virus At 24 hours after the inoculation a high virus concentration was revealed in the brain and serum with increasing virus titres during the next two days and persistence of a high level up to the death of the animals The spleen did not reveal any virus at 24 hours after inoculation but then the titres increased during the next few days The interferon content in the various organs showed a fairly close correlation to the virus titres and high values were observed in the brain during the last few days in the course of infection

Course of Infection after Splenectomy

In order to throw further light on the importance of the spleen in the course of infection some experiments were performed with splenec

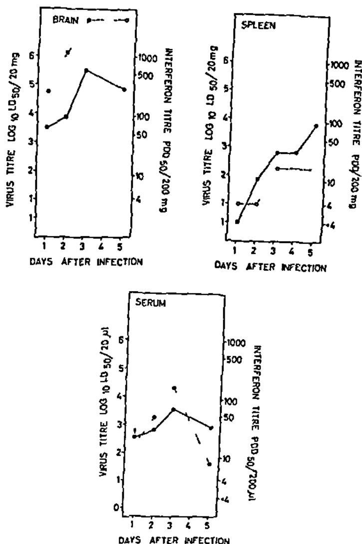


Fig 4

Titres of virus (●——●) and interferon (○——○) in the brain spleen and erum of 16–19 g mice inoculated intracerebrally with 100 intracerebral suckling mice l D$_5$ of West Nile virus causing death of 100 per cent of the infected mice

tomized mice As controls were used two groups of animals viz one in which sham operation had been performed and another which had not been subjected to surgery In order to approach the course of a natural infection as much as possible the inoculation was performed with the minimum infective dose of West Nile virus

Table 2 shows the titres of virus and interferon in the serum of the splenectomized animals and in the control groups As will be seen there was no distinct difference in the interferon levels in the three groups although there was a slight tendency to greater interferon

production in the splenectomized animals On the other hand the level of circulating virus was appreciably higher in the splenectomized group especially during the first few days after the inoculation In the experiments shown in the table no passage of virus to the brain was detected and no deaths occurred among the animals in this group In other experiments with inoculation of larger doses of virus passage of virus to the brain was observed earlier and in larger amounts in the splenectomized animals than in the other groups

TABLE 2

Titres of Virus and Interferon in the Serum of Splenectomized Sham Operated and Untreated Groups of Mice

Days after infection	Splenectomized Virus	Splenectomized Interf	Sham operated Virus	Sham operated Interf	No operation Virus	No operation Interf
2	1 9	<4	0 5	<4	0	<4
3	>3 5	4	2 3	<4	1	<4
4	3 5	6	2 4	4	1 3	4
6	2 7	4	0 9	<4	1 3	<4
8	<0	<4	0	<4	0	<4

DISCUSSION

The experiments reported here revealed the same sensitivity to the virus and fundamentally the same course of infection on subcutaneous intravenous and intraperitoneal inoculation However the course of the infection was not followed until 24 hours after the inoculation and it is possible that the response to the infection may have varied during the first few hours after the various routes of inoculation The uniform sensitivity and the subsequent uniform course of infection suggest that a local reaction if any occurring after subcutaneous or intraperitoneal inoculation is only of minor importance in the overall course of infection

The course of infection was different and the sensitivity was higher on intracerebral than on peripheral inoculation This seems reasonable in view of the fact that it was a neurotropic virus which was inoculated direct into the target organ A high titre of interferon was found in the brain after this mode of inoculation which is in agreement with previous reports (7 20) The finding of interferon and virus in serum and spleen after intracerebral inoculation differs from a report by *Subrahmanyan & Mims* (19) This might be explained by differences in mouse and virus strains

Intraperitoneal inoculation of small doses (1–3 LD_{50} as determined by intracerebral inoculation in suckling mice) induced subclinical infection in most of the animals A few mice became clinically ill but only in exceptional cases did the infection run a fatal course On administration of these doses we have thus a course of the infection

which may be compared to infection with neurotropic arbovirus in man On doubling the dose the infection led to death in most of the mice but only if the dose was about 100 times higher was the infection invariably fatal

The present experiments revealed a relatively high level of interferon in the spleen in an early phase after the administration of large infective doses The interferon titres were usually higher than the corresponding interferon titres in the serum This is in agreement with a study reported by *Van Rossum & De Somer* (21) who 6 hours after intravenous injection of Newcastle disease virus found interferon titres in the spleen which were 20 times as high as that in the serum In a previous study *Kono & Ho* (17) had reported that organs with reticulo endothelial cells responded rapidly to interferon inducing stimuli both *in vivo* and *in vitro*

In spite of the fairly uniform levels of viraemia the amount of circulating interferon was found to be considerably larger on the inoculation of large than on small doses

On inoculation of large doses the curve for circulating interferon was found to run roughly parallel with that for the interferon content of the spleen—a finding which may support the assumption of a correlation between circulating interferon and spleen interferon As the spleen was found to contain more interferon than the serum it will be reasonable to assume that passage of interferon occurs from the spleen to the serum and possibly also from other reticulo endothelial tissue and/or lymphoid tissue in the organism These observations provide evidence in support of the hypothesis advanced by *Van Rossum & De Somer viz* that the spleen is the most active interferon producing organ during viraemia (21)

On administration of the small infective dose only small amounts of circulating interferon were revealed and only small amounts were present in the spleen However the time relation of the occurrence of interferon in blood and spleen seemed to be less conspicuous here than after injection of the larger doses The interferon production in the spleen and the reticulo endothelial system may here play only a minor part In the experiments with splenectomized mice in which the dosage level was low the amount of interferon in the serum was of the same order of magnitude in the splenectomized animals and the controls In similar experiments in which large doses of Newcastle disease virus were used *Fruitstone et al* (10) found that the splenectomized mice had less interferon in the serum than the non splenectomized controls *Subrahmanyan & Mims* (19) had previously arrived at the same result on injection of influenza virus On the other hand *De Somer & Billau* (4) found the same amounts of interferon in the serum of splenectomized rats and control rats after injection of large doses of Sindbis virus

Interferon in the liver was demonstrated only in small amounts and

only after injection of large doses The virus titres for the liver were also low and it is not possible to assess whether independent infection of the organ with accompanying interferon induction had occurred or whether the interferon and virus demonstrated might have originated from residual blood in the organ

Demonstration of interferon in the brain was not possible until the organ was actually infected following which the interferon content was roughly correlated to the intensity of the infection of the organ Thus it was not possible to demonstrate passage of interferon from the serum to the brain in spite of the occurrence of fairly large amounts of circulating interferon in some of the experiments That such passage may occur in mice has been demonstrated in a number of experiments (3 8 9 14) in which protection against intracerebral inoculation of virus could be demonstrated when interferon had been injected intra venously or intraperitoneally before or simultaneously with the ino culation

The importance of interferon in the resistance of the host to and recovery from natural virus infection is a much debated problem which is scarcely fully clarified as yet However the balance between viraemia and circulating interferon may be conceived to play an im portant role in the outcome of the infection (3)

Disease and deaths among mice after infection with a neurotropic arbovirus seem to occur only in association with viral invasion of the central nervous system and here viraemia seems to play an important part (5) However the maximum viraemia observed in the experiments seems here to be only of minor significance as it was found to be fairly uniform in the three courses of infection studied in which a high level of viraemia was observed in the group of animals with only a few deaths

Early viraemia was observed in the groups with a subsequent high mortality It thus seems as if early onset of viraemia may be of im portance and that it may be the cause of the high mortality in the group subjected to large infective doses

With the infective doses and route of infection used it was not possi ble to reveal circulating interferon within the first 24 hours On the other hand later in the course circulating interferon could be demon strated after all infective doses Early viraemia developing after large infective doses together with the occurrence of no or only minor amounts of circulating interferon may be supposed to promote viral invasion of the central nervous system whereas the occurrence of circulating interferon later in the course may possibly protect the brain against the equally high level of viraemia which later develops after small infective doses

SUMMARY

The course of infection and the sensitivity to West Nile virus in mice were found to be uniform after intravenous intraperitoneal and sub cutaneous inoculation Mice inoculated intracerebrally revealed a dif ferent course of infection and simultaneously a higher sensitivity

On intraperitoneal inoculation of doses which were 100 per cent 90 per cent and 10 per cent lethal interferon and virus were demonstrated in the serum spleen and brain of the mice The highest levels were found on administration of the largest infective doses On the high doses interferon in the spleen occurred early with a titre which ex ceeded those obtained in the serum In addition fairly good parallelism between the curve for circulating interferon and that for the interferon content of the spleen was observed Small infective doses did not give rise to changes in the level of circulating interferon after splenectomy

The peaks of viraemia were fairly uniform in the three groups but viraemia occurred early in the groups with high mortality and it is suggested that a high mortality occurs in mice in which an early viraemia is demonstrable where no or only minor amounts of circu lating interferon are present while circulating interferon later affords protection of the central nervous system during a high level viraemia

REFERENCES

1 *Baron S & Buckler C E* Circulating interferon in mice after intravenous injection of virus Science *141* 1061-1063 1963
2 *Baron S et al* Relationship of interferon production to virus growth in vivo Proc Soc Exper Biol & Med *117* 338-341 1964
3 *Baron S et al* Role of interferon during viremia II Protective action of circulating interferon J Immunol 96 17-24 1966
4 *De Somer P & Billiau A* Interferon production by the spleen of rats after intravenous injection of Sindbis virus or heat killed Escherichia Coli Arch ges Virusforsch *19* 143-154 1966
5 *Downie A W* Pathways of virus infection Mechanisms of virus infection Edited by W Smith Academic Press London and New York 101-15 1963
6 *Fantes K H* Purification concentration and physico chemical properties of interferons Interferons Edited by N B Finter North Holland Publishing Company Amsterdam 119-180 1966
7 *Finter N B* A rich source of mouse interferon Nature (London) *201* 1114-1115 1964
8 *Finter N B* Protection of mice by interferon against systemic virus infections British M J *II* 981-985 1964
9 *Finter N B* Interferon as an antiviral agent in vivo Quantitative and temporal aspects of the protection of mice against Semliki Forest virus Brit J Exper Path *47* 361-371 1966
10 *Fruitstone M J et al* Role of the spleen in interferon production in mice Proc Soc Exper Biol & Med *122* 1008-1011 1966
11 *Gresser I & Dull H B* A virus inhibitor in pharyngeal washings from patients with influenza Proc Soc Exper Biol & Med *115* 192-195 1964
12 *Gresser I & Naficy K* Recovery of an interferon like substance from cerebrospinal fluid Proc Soc Exper Biol & Med *117* 285-289 1964
13 *Haahr S* The occurrence of interferon in the cerebrospinal fluid in patients with bacterial meningitis Acta path microbiol scandinav *73* 264-274 1968

14 *Hitchcock G & Isaacs A* Protection of mice against the lethal action of an encephalitis virus British M J *II* 1268–1270 1960
15 *Hitchcock G & Porterfield J S* The production of interferon in brains of mice infected with an arthropod borne virus Virology *13* 363–365 1961
16 *Isaacs A & Hitchcock G* Role of interferon in recovery from virus infections Lancet *II* 69–71 1960
17 *Kono Y & Ho M* The role of the reticuloendothelial system in interferon formation in the rabbit Virology *25* 162–166 1965
18 *Nagano Y & Kojima Y* Inhibition de l'infection vaccinale par un facteur liquide dans le tissu infecté par le virus homologue C R Soc Biol (Paris) *152* 1627–1629 1958
19 *Subrahmanyan T P & Mims C A* Fate of intravenously administered interferon and the distribution of interferon during virus infections in mice Brit J Exper Path *47* 168 176 1966
20 *Vainio T et al* Production of interferon by brains of genetically resistant and susceptible mice infected with West Nile virus Virology *14* 385–387 1961
21 *Van Rossum W & De Somer P* Some aspects of the interferon production in vivo Life Sciences *5* 105–113 1966
22 *Wheelock E F* Interferon in dermal crusts of human vaccinia virus vaccinations possible explanation of relative benignity of variolation smallpox Proc Soc Exper Biol & Med *117* 650–653 1964
23 *Wheelock E F & Sibley W A* Interferon in human serum during clinical viral infections Lancet *II* 382–385 1964

Acta path microbiol. scandinav 74 458–459 1968

BRIEF REPORT

RESPIRATORY SYNCYTIAL VIRUS COMPLEMENT FIXING ANTIBODIES

Evidence of the 7S Nature of Antibodies Determined with Dextran Added to the Test System

By *Allan Hornsleth*

The complement fixation (CF) test is only of limited value for the diagnosis of respiratory virus infection in infancy and early childhood (3 9 12) Only a part of the diseased children from whom virus can be isolated develop a significant antibody response as revealed by the CF test

Previous Experiments

It has been described that the diagnostic capacity of the CF test can be substantially improved by addition of the polysaccharide dextran to the reaction mixture (6) Dextran T 80 (mean molecular weight of 80 000) in a concentration of 10 per cent (g/100 ml) was added to the mixture of complement antigen and test serum (6) The CF technique of *Bradstreet & Taylor* (2) was employed with some modifications and the diluent (veronal buffered saline) was used with increased salt concentrations (6) When sera from infants and children with acute respiratory disease were tested with respiratory syncytial (RS) virus and para influenza virus CF antigens the titres found in tests supplemented with dextran (CF d titres) were in most cases higher than CF titres found in tests without dextran (CF v titres) Furthermore in some cases significant titre increases could only be found in tests with dextran (6) It was suggested that dextran in some way increased the activity of the antibodies in the sera examined but the kind of antibodies which were possibly activated was not established The findings presented in this paper suggest that dextran increases the activity of some 7S antibodies

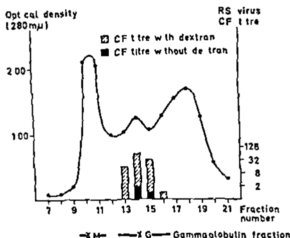
Present Experiments

Sera showing presence of antibodies against RS virus CF antigen both with and without dextran incorporated in the reaction mixture have been fractionated on a Sephadex G 200 column The fractions harvested have been tested with antihuman γG (7S) and γM (19S) antisera by gelprecipitation (double diffusion) in agarose and have at the same time been tested for CF d and CF v antibodies against RS virus

0 25 ml of undiluted serum was layered on top of a Sephadex G 200 column which measured 25 cm in length and 1 cm in diameter A 0 9 per cent sodium chloride solution was used as the eluent and 1 0 ml fractions were harvested and their optical density determined at 280 mμ in a Carl Zeis PMQ II spectrophotometer 0 025 ml volumes of the fractions were at the same time tested by the Ouchterlony technique in agarose layers of 2 6 mm thickness against 0 025 volumes of goat antihuman 7S gammaglobulin serum and goat antihuman 19S gammaglobulin serum The agarose was obtained from l Industrie Biologique Francaise S A and used in a concentration of 1 per cent in isotonic veronal buffered saline (VBS) from Oxoid Ltd England The anti 7S and anti 19S sera were obtained from Immunology Inc Illin is U S A When the fractions were examined in CF tests against RS virus antigen the technique of *Bradstreet & Taylor* (2) was employed with some modification as described previously (6) The RS virus CF antigen was also the same as used previously (5) The dextran employed in some of the CF tests was dextran T 40 (mean molecular weight 40 000) obtained from Pharmacia Ltd Sweden It was used in a concentration of 10 per cent in the mixture of complement antigen and serum and with a dilution of the diluent employed (VBS) which gave a sodium

Received 20 ix 68 *from the Institute of Medical Microbiology University of Copenhagen Denmark*

The technical assistance of Miss *Hanne Ekelöf* is gratefully acknowledged



Gel filtration on Sephadex G 200 of a serum sample from a 4 year old patient with acute bronchitis Distributions of densities and RS virus CF titres in the eluted fractions are shown The fractions showing content of 7S or 19S globulines by gelprecipitation are also indicated

chloride concentration of 1 28 per cent in the mixture Dextran T 40 and dextran T 80 can both be used with equal success for improving the CF test with RS virus antigen

The figure shows the results obtained by fractionation of a serum sample from a 4 year old patient with acute bronchitis This serum had a CF d titre of 1024 and a CF v titre of 64 Only the fractions showing presence of 7S antibodies reveal CF activity Analogous experiments with other sera containing RS virus CF d and CF v antibodies gave similar results

Discussion

In experimental infections with different kinds of viruses CF antibodies have been found to be almost entirely 7S globulin while 19S globulin has shown only a low ability to fix complement (8) It was suggested as a possibility that dextran increased the CF activity of 19S antibodies in virus infections in infants and children (6) This possibility must be ruled out by the findings presented in this paper Also CF antibodies to adenovirus tumour neoantigen has been shown to be of the 7S nature (11) while CF antibodies to *M pneumoniae* may be both 7S and 19S immunoglobulines (1)

It should be noted that also the activity of virus neutralizing antibodies can be increased by the addition of different protein factors to the test system Unheated guinea pig serum increases the activity of neutralizing antibodies to herpes virus (4) and to rubella virus (7) whil antibody cofactor" can activate neutralizing antibodies to influenza virus especially antibodies of the 7S nature (10)

References 1 *Biberfeld G* J Immunol *100* 338–347 1968 —2 *Bradstreet C M P* & *Taylor C E D* Mth Bull Minist Hlth Lab Serv *21* 96–104 1962 —3 *Chanock R M Mufson M A & Johnson K M* Progr med Virol 7 208 252 1965 —4 *Hampar B Notkins A L Mage M & K ehn M A* J Immunol *100* 586–593 1968 — 5 *Hornsleth A* J Path Bact 92 210–217 1966 —6 *Hornsleth A* Proc Soc. Exp Bi l Med in print 1968 —7 *Leerhøy J* Acta path microbiol scandinav 73 275–8? 1968 —8 *Pike R M* Bact Rev *31* 157–174 1967 —9 *Portnoy B Salvatore M A Hanes B Hammer D J Lee lom J M Jamba ian A & Eclert H L* Amer J Epid *86* 36? 371 1967 —10 *Styl B & Hana L* Acta virol 9 109–118 1965 —11 *Van Hooster Jr C L Cupp C Boren D & Trentin J J* J Immunol 100 128–132 1968 —12 *Va gosko A J Kim H W Parrott R H Jeffries B C Wong D & Chanock R M* Bact. Rev 29 487–495 1965

These experiments establish definitely that the inoculum was in fact *Toxoplasma* positive and that the results obtained were not due to already existing infections in the mice which were used

The remaining factor to be determined was the absence of *Toxocara* ova from the inoculum 12 drops of the undiluted inoculum were microscopically examined on slides and found to be *Toxocara* negative The slides were then washed into a beaker and the washings concentrated by centrifugation This concentrated material was orally administered to 6 dye test negative mice A further 6 mice were not subjected to any experimental procedures and were set aside these controls were both serologically and parasitologically negative when examined subsequently Only 2 of the 6 mice fed with the washings survived to autopsy 47 days later both were positive for *Toxoplasma* serologically having a dye test titre of 1 1250 Cysts could be observed in the brains of both mice The 4 mice which died were also infected with *Toxoplasma* because when their brains were inoculated into groups of dye test negative mice infections could be demonstrated in all passage animals As a result of this experiment we concluded that *Toxoplasma* could be faecally transmitted in the absence of nematodes

We concede that these results are based on an inoculum prepared from the faeces of a single cat and that this was the only positive result from 17 *Toxocara* negative individuals However there can be no doubt regarding the toxoplasmic positivity of this inoculum as the protozoan has been isolated from it on many occasions by mouse passage More recently we have been successful in repeating the above results We have obtained *Toxoplasma* positive faeces from 1 of a further series of 4 *Toxocara* negative cats using similar techniques to those described above In addition we must remember that *Jacobs* (1967) has reported that he has observed 2 similar cases

We have had many successes in transmitting toxoplasmosis in the faeces of *Toxocara* positive cats and few with *Toxocara* negative cats In view of this and of the micro isolation experiments of *Dubey* (1967) we feel that the question of nematode transmission is still open However in addition to this possibility we have now clearly demonstrated that *Toxoplasma* infections can be faecally transmitted in the absence of nematodes We are convinced that there are unknown forms of *Toxoplasma* which are passed independently in faeces and which are capable of surviving in moist conditions for at least 3 months

References *Dubey J P* J Protozool 14 Suppl p 42 1967—*Dubey J P* Veterinary Bulletin 38 495–499 1968—*Hutchison W M* Nature *206* 961–962 1965—*Hutchison W M* Trans R Soc Trop Med Hyg *61* 80–89 1967—*Jacobs L* Advances in Parasitology 5 1–45 1967—*Sabin A B* & *Feldman H A* Science *108* 660–663 1948

Acta path microbiol scandinav 74 465-486 1968

The Wallenberg Laboratory University of Uppsala Uppsala Sweden

LONG TERM CULTURE OF NORMAL AND NEOPLASTIC HUMAN GLIA

By

JAN PONTÉN[1] and ELIZABETH H MACINTYRE

Received 22 iv 68

The pioneer work by *Harrison* (1910) *Kredel* (1928) *Buckley* (1929) and *Russell & Bland* (1933) established that central nervous system (CNS) tissues would grow successfully *in vitro* Subsequent studies (*Cox & Cranage* 1937) *Pinkus* 1937 *Costero & Pomerat* 1955 *Lumsden* 1959 *Manuelidis & Pond* 1959 *Shein* 1965 1967) used this technique principally as a means of extending morphological observation from histologic sections to the level of the single living cell with special emphasis on histogenetic problems For this purpose primary explants have been the material of choice

The aim of the present work was the isolation of glial cell lines[3] of both benign and malignant origin and their subsequent comparison with regard to morphology and capacity for prolonged growth *in vitro* The emphasis has been on the behaviour of stabilized long term cultures rather than the primary short term outgrowth In contrast to previous experiments which have dealt with isolated selected tumours this work encompasses a consecutive series of biopsies in order to ascertain the proportion of successful cultures obtained under routine conditions

Human fibroblast like cells of normal origin (*Hayflick & Moorhead* 1961) form stable lines[3] with a finite life span and do not undergo any spontaneous transformation to established lines with infinite growth potential Whether human glia shares this stability has not been determined Solid human neoplasms of epithelial and mesenchymal

---

[1] Department of Pathology University of Uppsala Uppsala Sweden

[2] Pathology Department University of Colorado Medical Center Denver Colorado 80220 USA

This investigation was supported by grants from *Damon Runyon Memorial Fund* *Jane Coffin Childs Memorial Fund* the *Swedish Cancer Society* the *Swedish Medical Research Council* and the *Ollie Engkvist Foundation* Sweden

[3] The original terminology of *Hayflick & Moorhead* (1961) distinguishing between strains with a limited life span and lines with an indefinite growth potential has here been abandoned in favour of the terminology adopted by the American Tissue Culture Association The word cell lines is used for any serially cultivated culture and the term established (cell)line for a population which is indefinitely propagable *in vitro*

origin have been notoriously difficult to cultivate Only a small percentage of such tumours will grow successfully *in vitro* (*Moore & Koike* 1964 *Ponten & Saksela* 1967) This report will demonstrate that human glial tumours have a higher capacity for growth outside of the body and describes four lines established from malignant gliomas

MATERIAL AND METHODS

Cell cultures Non malignant glial tissue came from wound débridement or was removed as part of the routine operation on haematoma or aneurysm Glioma tissue came from biopsies taken for histo pathological *diagnosis Grid cultures and* direct cell suspensions (usually two of each) were prepared on each case within twenty minutes of operative removal

Grid cultures employed several 1 2 mm fragments of tissue which were placed directly on top of wide meshed lens paper or of the gelatin foam Spongostan[1] which in turn covered a stainless steel grid (*Jensen et al* 1964 *Ponten & Saksela* 1967) The grids lay in 50 mm Falcon plastic Petri dishes whose fluid level was at the upper surface of the grid Cells grew out from the explants into the interstices of their support Some cells detached from the explant and reattached to the bottom of the culture vessel where they formed cell layers Grids were transferred to a fresh plate when cells covered the area underlying the grid (approximatel 2 cm^2) *Cell transfers from plates deriving from grids were performed when the bottom* was covered by cells using 0 25 per cent trypsin and a split ratio of 1 2

Direct cultures were prepared by seeding small fragments of the specimen broken up by vigorous pipetting Subsequent cell transfers followed the method for grid cell transfers For staining coverslip were placed in freshly seeded Petri dishes Eagle s minimum essential medium (MEM) with 10 per cent uninactivated calf serum 100 units of penicillin/ml and 50 μg of streptomycin/ml was used Medium change was made biweekly Tissues were grown at 37 C in a humidified atmosphere containing 5 per cent CO in air

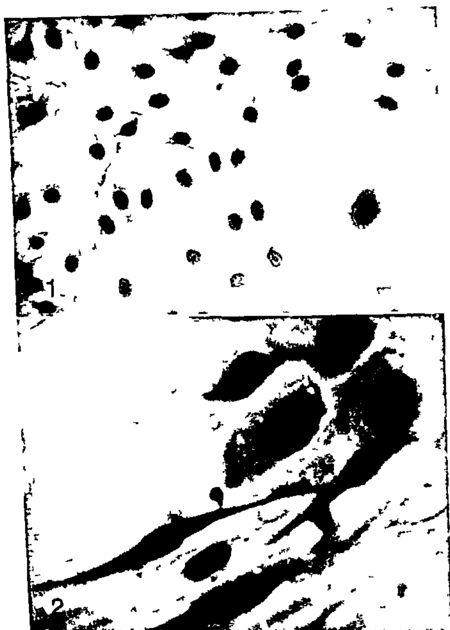
Histological preparations The initial diagnostic biopsies tissues on Spongostan grids and cell blocks prepared by trypsinizing cell layers and centrifuging out the *cells were fixed in 10 per cent formalin overnight Coverslips and whole plates were* fixed by methyl alcohol acetic acid (3 1) for one hour Brom formalin fixation was used in the Cajal or del Rio Hortega procedures Haematoxylin and eosin haematoxylin and van Gieson May Grunwald Giemsa Cajal s gold chloride Penfield s second modification of the del Rio Hortega silver carbonate Mallory s phosphotungstic acid haematoxylin (PTAH) and Gomori s reticulin were used *The Cajal Penfield modification* of del Rio Hortega and PTAH were used to differentiate *astrocytes from microglia* oligodendroglia and spongioblasts (*Penfield & Cone* 1950 *Shein* 1965) The PTAH stain distinguishes the slender astrocytic fibrils from

Figs 1–2

Fig 1 Near-confluent sheet of astrocyte like cells from non neoplastic brain tissue The field is dominated by monomorphic 2nA astrocytic cells which have oval nuclei and barely discernible cytoplasmic fibrils To the right a 4nA tetraploid astrocytic *cell with a large oval nucleus* The voluminous cytoplasm has in parts been *retracted* due to fixation artefacts May Grünwald Giemsa Appr magn × 400

Fig 2 Cells *from line* 125 CC derived from non neoplastic brain tissue 5 nuclei from 2nA astrocytic cell are seen The lower half of the picture shows three spongioblasts (*S-cells*) *with their characteristic* bipolar shape and lean nuclei The S-cells are located on top *of the* 2nA cells and therefore slightly out of focus May Grunwald Giemsa *Appr magn* × 1000

1 Obtained from Ferrosan Malmo Sweden


1
2

the coarse fibres of fibrous and muscle tissue Gomori's reticulin impregnation is widely used to differentiate cells containing reticulin from microglial cells (Marshall 1956 Person & Voris 1965 Samuelsson et al 1966) In our hands the Cajal and del Rio Hortega were difficult to use on monolayers and proved much more reproducible on blocks of tissues PTAH was very reliable on monolayers as well

Exposure of glial cultures to RSV(FH) Four day old glioma derived cultures were exposed to 0 1 ml of Rous sarcoma virus of the Engelbreth Holm strain (RSV(FH)) for one hour at 37 C at a multiplicity of about 1 after removal of medium The cells were then washed twice with MEM and left in fresh MEM The virus exposed cultures together with non exposed cultures of otherwise identical history were studied daily for periods up to six months Infectivity tests were carried out by exposing chicken fibroblasts to supernatant fluid from transformed cultures The standard method described by Rubin (1960) was followed

Tests in chickens for induction of tumours by Rous exposed glial cells One glioma line transformed by RSV(EH) was harvested with trypsin and injected into the wing web of 16 ten day old leucosis virus free chickens using 0 9 × 10⁶ cells per site The injection site was checked for tumour formation for six weeks

Exposure of normal glia to glioma tissue culture fluid Three day old medium from several plates of glioma cells were combined centrifuged at 2000 rpm for 15 minutes and the supernatant added either to a freshly explanted control glia grid or to mitosing control glial cells in serial passage In all eight primary and three secondary control cultures were challenged After two hours incubation at 37 C the plates were rinsed twice with MEM and the cells refed with growth medium In one case the centrifuged fluid was put through a 0 45 μ millipore filter prior to use The exposed cultures were observed for three months

Cell enumeration An automatic Celloscope[1] was used All counts represent the average of at least four counts two from each of duplicate plates

RESULTS

Terminology

2nA cells (Astrocytic cells) (*Costero & Pomerat 1955 Shein 1965*) These have a single oval normochromatic nucleus (Fig 1) a small nucleolus and abundant palely basophilic cytoplasm whose periphery often extends as elaborate ramifications Gliofibrils were impregnated by Cajal's gold chloride and Mallory's PTAH but not by Gomori's reticulin or Penfield's modification of the del Rio Hortega silver carbonate stain

4nA cells (*Tetraploid astrocytic cells*) The general morphology resembles that of the 2nA cells but the nucleus is about twice as big and carries 1–3 nucleoli The cytoplasm is abundant moderately basophilic and contains neurofibrils (Fig 1)

S cells (Spongioblasts) (*Costero & Pomerat 1955 Shein 1965*) These cells commonly appear on top of astrocytic cells singly or in clusters (Fig 2) They have a tiny hyperchromatic oval nucleus a narrow rim of cytoplasm usually in bipolar arrangement with a neuroglial net work (*Costero & Pomerat 1955*) which stains well with Penfield's modification of del Rio Hortega's stain but not with PTAH or Cajal's gold chloride

MG cells (*Atypical cells from gliomas*) (Fig 3) The nucleus usually single may be multiple It is hyperchromatic and has a sharply

---

[1] Supplied by AB Lars Ljungberg Stockholm Sweden

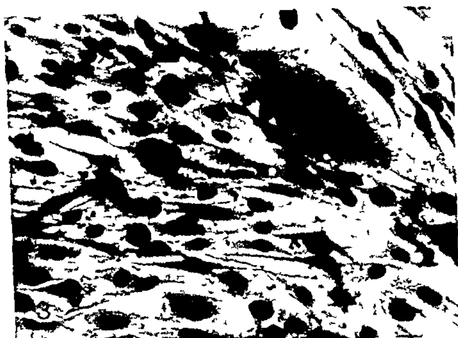


Fig 3

Cells from the 5th passage of line 135 MG-B The lines of group MG-B and MG-C showed individual characteristics particularly in their early passages They were all, as illustrated in this figure distinguished from CG and MG-A lines by an increased cellularity pleomorphism and the common occurrence of multinucleated cells examples of which are seen near the centre and in the upper right part of the field Note presence of a few spongioblasts (lower right) which do not differ in appearance from those seen in control cultures (Fig 2) In spite of a high initial growth rate and an enhanced capacity for serial passage cultures of series MG-B such as the illustrated example eventually entered a degenerative phase and were lost May Grunwald Giemsa Appr magn × 400

defined border often interrupted by protrusions and irregular indentations Multilobed variants are sometimes frequent Mitoses are more common than in cultures of normal glia and are sometimes grossly abnormal Associated cytoplasm is of extremely variable morphology between tumours but may show some constancy within the same tumour It is often voluminous and deeply basophilic with sharply defined borders which often arborise Neuroglial content is highly variable (Fig 4)

Description of Cultures

1 *Cultures of non neoplastic glia (CG lines)* Specimens from 14 cases were cultured Females were in preponderance Growth was obtained in all cultures Although initial growth was slower from the grids than from the direct cultures both produced the same range of cells which grew at the same rate to the same terminal density and

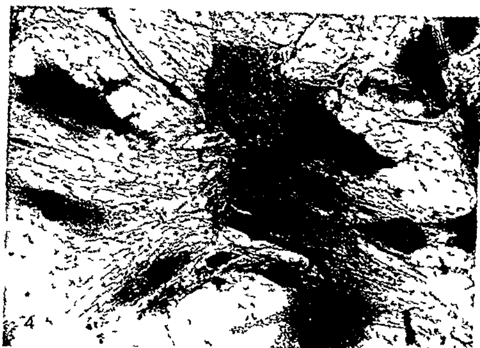


Fig 4

Atypical MG cells from line 165 MG-B in passage 8 Note the rich network of neuro fibrils The fibril content varied from culture to culture of the MG-B and MG C series With time the fibrils tended to disappear Phosphotungstic acid haematoxylin Appr magn 1 600

yielded equivalent numbers of transfers for each case The subsequent description will make no distinction between grid and direct primary cultures

The earliest outgrowth usually seen after 4-6 days consisted of astrocytes Further growth was slow and continued till a monolayer

---

Figs 5-6

Fig 5 Line 153 CG in passage one at maximal cell density Note preponderance of 2nA cells arranged in a non descript pattern Only two 4nA cells are seen There is no nuclear overlapping and mitotic activity is absent May Grunwald Giemsa Appr magn × 120

Fig 6 Line 1 3 CG in passage 13 which was one passage before the culture was l t at the end of its finite life span *in vitro* (phase III) *The cell density is* lower than during the stage of rapid growth (phase II) *Compare with Fig 1* The relative volume of the cytoplasm is greatly increased with an elaborate network of fibrils of varying caliber The nuclei are pleomorphic with one multilobed in the upper part of the picture Phase III normal glia cells resemble the cells derived from malignant gliomas by their pleomorphism but differ in being normo or hypochromatic having small nucleoli and virtually lacking mitotic figures May Grunwald Giemsa Appr magn × 400

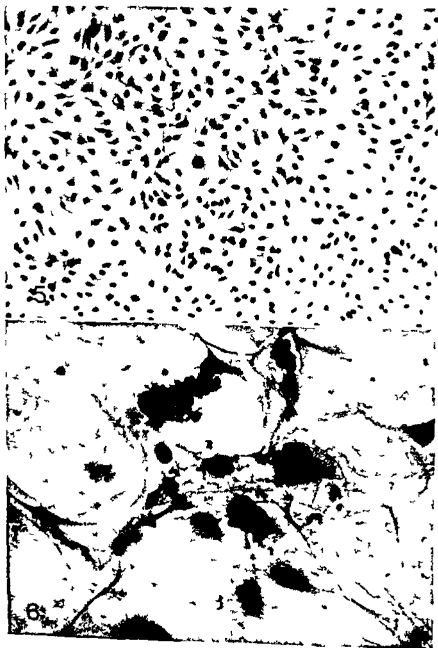

5
6

TABLE 1

Growth Characteristics of Cultures from Non Neoplastic Glia and Gliomas

Type	n	Time until first transfer (days)				Termination		
		Grid cult		Direct seed cult		Max no transf		Cause of term
		Mean	Range	Mean	Range	Mean	Range	
CG	10	35.6	23–48	24.9	13–38	15.9	10–24	phase III
MG A	10	33.2	25–41	19.2	14–26	14.8	8–22	phase III
MG B	15	14.0	11–33	12.1	9–24	22.4	11–31	phase III
MG C	4	15.1	13–19	14.7	7–18	Apparently infinite		

CG = cultures derived from non neoplastic glia
MG = cultures derived from gliomas
MG A = cytologically indistinguishable from group CG
MG B = cytologically atypical Finite life span
MG C = cytologically atypical Infinite life span

covered the dish (Fig 5) This required 5–7 weeks During the latter part of this period S cells were identified in some cultures (Fig 2) Phagocytic cells often multinucleated appeared at a variable time from explantation No other cell types were found in normal cultures Mitoses were infrequent and ceased after monolayer formation The interval between transfers during phase II[1] growth varied between averages of 15 and 26 days In this period the distribution of 2nA 4nA S cells was usually in the range 94 5 1 S cells tended to diminish in number on repeated passage The output from grids usually fell off after 3–4 months Histological section of the grid tissue at this time (discussed further in Section 3) showed no surviving glial cells

Phase II lasted till about 4–12 months after explantation when phase III[1] began The maximal average life span corresponded to 15.9 transfers (Table 1) The percentage of 4nA cells rose during phase III and giant nuclei often with lobulated nuclear protrusions appeared Pyknosis increasing nuclear pleomorphism and bizarre forms with extensive tortuous cytoplasmic extensions became increasingly frequent among the astrocytes (Fig 6) S cells which still persisted in small numbers in some cultures resembled those of phase II The cell density decreased as exemplified by line 158CG which reached a terminal cell density of 50 000 cells/cm² at transfer 7 12 000 at 13 and 8 000 at 15 Attempts to improve viability by pooling phase III cultures failed

2 *Cultures of malignant glial tissue (MG lines)* The cultures originated in adult humans whose average age was 49 years (range 21 73 years) There was a slight preponderance of males

1 Phase II is defined as the period of serial passage when growth in maximal and degenerative changes insignificant phase III as the period of decreasing net growth rate preceding eventual complete degeneration (*Hayflick & Moorhead 1961*

TABLE 2

Histology and Tissue Culture Grouping of Lines from Malignant Gliomas

Identification	Histological diagnosis (Ringertz classification glioma)		Salient histological features
83 MC A	Glioblastoma	(grade III)	Cysts
88 MC A	Glioblastoma	(grade III)	OD
95 MC A	Astrocytoma	(grade I)	Malignancy questionable
101 MC A	Oligodendroglioma	(grade II)	GA Pleomorphism
108 MG A	Astrocytoma	(grade II)	Isomorphous
120 MC A	Glioblastoma	(grade III)	Necrosis
127 MC A	Glioblastoma	(grade III)	Necrosis
131 MC A	Astrocytoma	(grade II)	NF
166 MC A	Glioblastoma	(grade III)	Pleomorphism
175 MC A	Glioblastoma	(grade III)	Necrosis
66 MC B	Glioblastoma	(grade III)	GA
67 MC B	Glioblastoma	(grade III)	GA NF
70 MC B	Glioblastoma	(grade III)	GA fibrosis groups of rounded cells
73 MC B	Glioblastoma	(grade III)	Groups of small rounded cells
104 MC B	Glioblastoma	(grade III)	Pleomorphism
135 MC B	Glioblastoma	(grade III)	NF necrosis
136 MC B	Glioblastoma	(grade III)	Necrosis fibrosis
144 MC B	Glioblastoma	(grade III)	
147 MC B	Glioblastoma	(grade III)	Pleomorphism necrosis
150 MC B	Glioblastoma	(grade III)	Anaplasia
161 MC B	Glioblastoma	(grade III)	Anaplasia
165 MC B	Glioblastoma	(grade III)	
168 MC B	Glioblastoma	(grade III)	NF necrosis GA
170 MG B	Glioblastoma	(grade III)	Spongioblasts
174 MC B	Glioblastoma	(grade III)	Spongioblasts
87 MC C	Glioblastoma	(grade III)	Necrosis large tumour cells
10 MC C	Glioblastoma	(grade III)	Extreme pleomorphism
118 MC C	Glioblastoma	(grade III)	Pleomorphism fibrosis
138 MC C	Glioblastoma	(grade III)	OD
119 MC D	Glioblastoma	(grade III)	Lymphocytic perivascular cuffing

MC A = quasi normal
MC B = atypical cells—finite life span *in vitro*
MC C = atypical cells—infinite life span *in vitro*
MC D = glioma showing lymphoblastoid transformation
NF = neurofibrils
GA = gemistocytic astrocytes
OD = oligodendroglia

(i) *Histology of surgical biopsies* There were 30 astrocytomas glioblastomas which were graded according to the Ringertz system (*Ringertz* 1950)[1] and one oligodendroglioma (Table 2)

(ii) *Tissue culture characteristics* Satisfactory growth was obtained in 30 cases Tissue received from the 31st (a glioblastoma) was mainly necrotic and was

[1] *Ringertz* (1950) classification recognizes three grades astrocytoma intermediate type and glioblastoma These are identified by Roman numerals I II and III respectively in the text and tables

lost in early passage The other lines were classified in the following way MG A quasi normal cultures MG B pathological cultures—finite life span *in vitro* MG C pathological cultures—indefinite life span *in vitro* and MG D the unique line 119 MG showing lymphoblastoid transformation (*Benyesh Melnick et al* 1963)

A *Quasi normal cultures* One oligodendroglioma and all grades of astrocytoma were represented among the ten cases All three astrocytomas of grade I and II were found among the quasi normal cultures (Table 2) No difference in growth rate interval between transfers or total *in vitro* life span was observed between the quasi normal cultures and cultures derived from non neoplastic brain tissue (Table 1) They all terminated in phase III status The cultures deriving from this group were indistinguishable from normal glial cultures after serial passage Occasionally large anaplastic astrocytes stood out against a background of normal glia in the first few passages

B *Pathological cultures—finite life span in vitro* This group encompassed 15 glioblastomas (Table 2) The primaries plated out quickly and subcultivation could be started after an average of 12 1–14 0 days (Table 1) The cell population was dominated by MG cells from the beginning Both within and between the different cases these cells varied greatly in morphology orientation with respect to each other and relative differentiation of nucleus and cytoplasm (Fig 3) MG cells grew in an irregular untidy highly variable pattern whose salient features were haphazard orientation and pleomorphism *Mitosis* continued in multilayers of cells and the mitoses were often abnormal Phagocytic cells were found in small numbers Spongioblasts were more frequent than in controls or quasi normals and were of apparently normal morphology In several cultures there were islands of mitosing small cells with a single rounded hyperchromatic nucleus and scant cytoplasm These cell nests were morphologically identical to those described later in 118 MG C cultures Debris was present throughout phase II and onwards in most cultures The terminal cell density (at least 90 000 cells/cm²) was higher than that of the quasi normals or controls Some cell lines as 73 MG grew very quickly and were transferred weekly during phase II Eventually however all lines entered phase III and were lost The final transfer number varied between 11–31 (average 22 4) and was thus higher than among most of the controls or the quasi normals No permanent cell lines were obtained (Table 1)

C *Pathological cultures—indefinite life span in vitro* This group included lines of variable appearance—87 MG 105 MG 118 MG and 138 MG—all derived from highly malignant astrocytomas (Table 2)

87 MG-C was cultured from a grade III astrocytoma glioblastoma in a 44 year old woman The MG cells were large extremely bizarre and grew very slowly (Figs 7 and 8) Maximal cell densities of about 90 000 cells/cm² were similar to those of group B but were less than those of

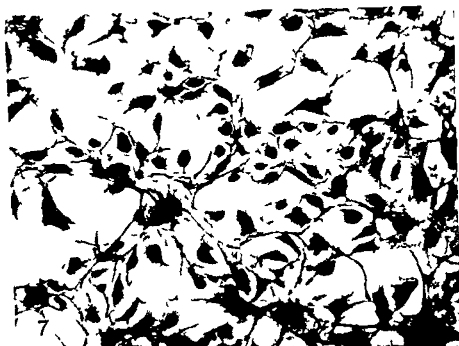


Fig 7

Line 87 MG in early passage Note pleomorphism local variations in cell density and irregular cytoplasmic ramifications May Gruwald Giemsa Appr magn × 400

105 MG C and 118 MG C Table 3 gives the intervals between subcultivation from the 3rd transfer onwards Growth crises occurred between the 3rd and 8th transfers and again around transfer 13 interpersed by spurts of rapid proliferation

105 MG-C originated from a grade III astrocytoma glioblastoma in a 62 year old woman The cells plated out quickly and proliferated vigorously The average interval between transfers has been 12 days A profusion of pathological astrocytes (MG cells) (Fig 9) the display of varied cell patterns and the abundance of morphologically normal spongioblasts characterized this line Mitoses were frequent even at high cell densities Neurofibrils were plentiful in immature cells in others the cytoplasm was packed with orange red granules (Fig 10)

TABLE 3

Transfer Intervals for Line 87 MG C

Transfer	—3	—4	—5	—6	—7	—8	—9	—10	—11	—12	—13	
	38	7	85	84	146	10	1[illegible]	8	14	104	53	Days
Transfer	—14	—15	—16	—17	—18	—19	—20	—21	—2	—23	—24	
	11	7		10	[illegible]							

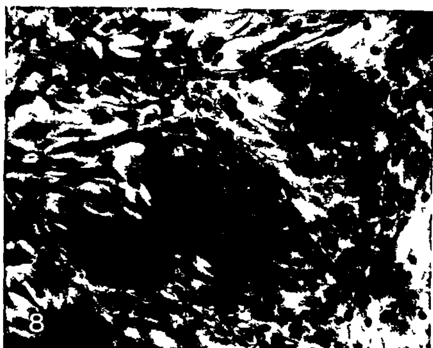


Fig. 8.

Line 87 MG-C in passage 32. The cell density has increased considerably compared to the early passage illustrated in Fig. 7, but this particular line still shows only little tendency to growth in multilayers. The cells have assumed a rounded or polygonal form with loss of most of the cytoplasmic ramifications and fibrils. May-Grunwald-Giemsa. Appr. magn. × 200.

(May-Grunwald-Giemsa). In some cultures S cells provided up to 50 per cent of the population, lay as dense tangles on top of the MG cells and were not diminished by frequent transfers. A large amount of débris was observed which did not disappear when the cells were grown in the presence of kanamycin or aureomycin. 105 MG-C is at present in its 68th transfer with a maximal density of about 150 000 cells/cm².

118 MG-C came from a grade III astrocytoma-glioblastoma in a 50-year-old man. One area of the tumour resembled ependymoblastoma. The cells have a 15 day average transfer interval in culture. The cellular pleomorphism and pattern variety in tissue culture were reminiscent of 105 MG-C. Cytoplasmic granulation was even more striking than in 105 MG-C, while bizarre tumour astroblasts were frequent and often included a wealth of neurofibrils. Spongioblasts were abundant, appeared normal and were not adversely affected by frequent subcultivation. 118 MG-C has now reached its 52nd transfer and continues to grow luxuriantly more than one year after explantation. Its maximal cell densities are equivalent to those of 105 MG-C.

138 MG-C originated from a grade III astrocytoma-glioblastoma



Figs 9–10

Fig 9 Line 10 MC C in passage 29 Note strong tendency of cells to grow as irregular clumps(lower left corner) Many of the cells radiating out from the clump have the appearance of bipolar spongioblasts which were numerous in this particular line May Grunwald Giemsa Appr magn × 1 900

Fig 10 Line 10b MC-C in passage 19 Numerous acidophilic granules of unknown nature are seen in the cytoplasm of many cells Similar granules may also be seen in a few cells depicted in Fig 9 May Grunwald Giemsa Appr magn × 1 600

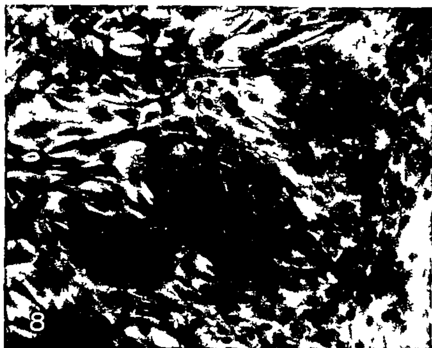


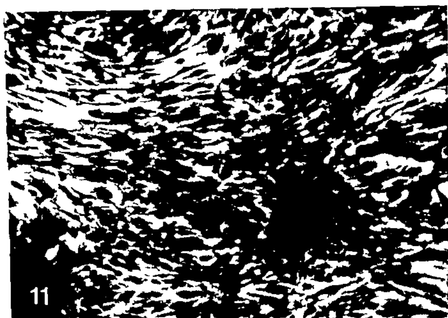
Fig 8

Line 87 MG-C in passage 32 The cell density has increased considerably compared to the early passage illustrated in Fig 7 but this particular line still shows only little tendency to growth in multilayers The cells have assumed a rounded or polygonal form with loss of most of the cytoplasmic ramifications and fibril May Grunwald Giemsa Appr magn × 200

(May Grunwald Giemsa) In some cultures S cells provided up to 50 per cent of the population lay as dense tangles on top of the MG cells and were not diminished by frequent transfers A large amount of débris was observed which did not disappear when the cells were grown in the presence of kanamycin or aureomycin 105 MG-C is at present in its 68th transfer with a maximal density of about 150 000 cells/cm²

118 MG-C came from a grade III astrocytoma glioblastoma in a 50 year-old man One area of the tumour resembled ependymoblastoma The cells have a 15 days average transfer interval in culture The cellular pleomorphism and pattern variety in tissue culture were reminiscent of 105 MG-C Cytoplasmic granulation was even more striking than in 105 MG-C while bizarre tumour astroblasts were frequent and often included a wealth of neurofibrils Spongioblasts were abundant appeared normal and were not adversely affected by frequent subcultivation 118 MG-C has now reached its 52nd transfer and continues to grow luxuriantly more than one year after explantation Its maximal cell densities are equivalent to those of 105 MG-C

138 MG-C originated from a grade III astrocytoma glioblastoma


11


12

TABLE 4

Histological Comparison between Original Tissue and Explants Kept as Grid Cultures

Strain Identification (Table 2)	Original tissue diagnosis (Ringertz)	Diagnosis from grid tissue	Comments	Time from explantation	Seeding cells when sampled
153 CC	Not neoplastic	None	Degenerate	4 Mo	No
164 CC	Not neoplastic	None	Degenerate	3 Mo	No
127 MC A	Glioblastoma III	None	Degenerate	3 Mo	No
104 MC B	Glioblastoma III	Glioblastoma III	Tumour giant cells and astrocytes	7 Mo	Yes
104 MC B	Glioblastoma III	None	Degenerate	12 Mo	No
135 MG B	Glioblastoma III	None	Astrocytes	6 Mo	Yes
130 MC B	Glioblastoma III	None	Occasional GA vessel ghost GA (Fig 13)	6 Mo	No
147 MC B	Glioblastoma III	Astrocytoma II	Tumour giant cells GA	4 Mo	Yes
150 MC B	Glioblastoma III	Glioblastoma III	Tumour giant cells GA	3 Mo	Yes
16? MG B	Glioblastoma III	Glioblastoma III	Tumour giant cells astrocyte and macrophages	[illegible] Mo	Yes
170 MC B	Glioblastoma III	Small cell glioblastoma III	Well characterized tumour	1 Mo	Yes
174 MC B	Glioblastoma III	Oligodendroglioma II	OC vascular connective tissue	3 Wk	Yes
105 MC C	Glioblastoma III	Glioblastoma III	Tumour giant cells and GA (Fig 12)	12 Mo	Yes
118 MC C	Glioblastoma III	Glioblastoma III	Well preserved	1 Wk	Yes
119 MC D	Glioblastoma III	Glioblastoma III	See text	6 Mo	Yes
119 MC D	Glioblastoma III	Glioblastoma III	See text	6 Mo	Yes

S = Spongioblast GA = Gemistocytic astrocytes OC = Oligodendroglia

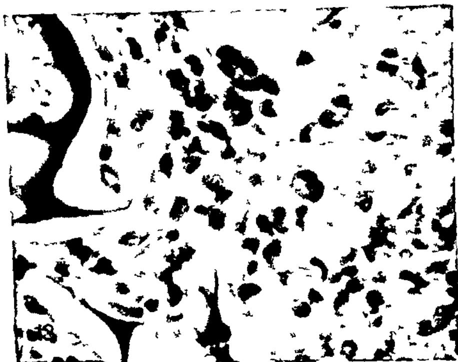


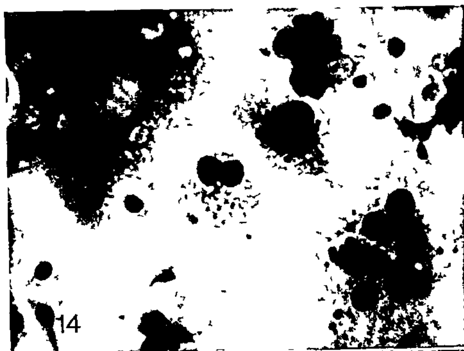
Fig 13

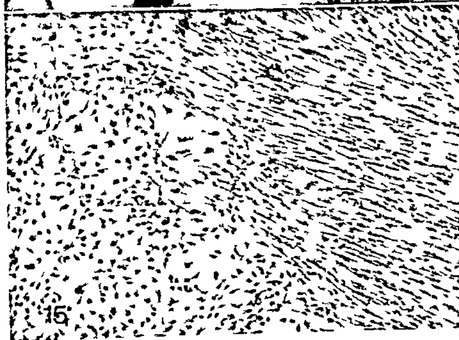
Section from one spongostan grid carrying an explant from which line 147 MG B was derived The original tumour was classified as a grade III glioblastoma with only slight admixture of gemistocytic astrocytes The grid sectioned after 4 months shows a preponderance of moderately pleomorphic astrocytes of gemistocytic type On the basis of the illustrated grid section the tumour was classified as a grade II astrocytoma Gelatin foam fibres are seen in the left part of the picture Van Gies n Appr magn × 1 000

The 119 MG-D grid which started to yield immunoglobulin producing cells after three months and a half was sectioned after a further 3 months Lymphoid cells could clearly be identified Such cells were not present in other grids which never yielded lymphoblasts

Exposure of Normal Glial Cultures to Supernatant from Group B and C Glioma Cultures

Supernatant from 11 fast growing pathologic tissue cultures of glioma originating in 67 MG 87 MG 105 MG and 118 MG were added to (a) freshly isolated normal glial tissue on grids and (b) proliferating normal glial cells in phase II In two cases where 118 MG supernatant was used the cells grew out faster than the control cells to which supernatant had not been added and an abnormal cell population developed Chromosome study however showed that the proliferating cells was of the same karyotype as the original tumour The experiment was repeated using centrifuged and Millipore filtered supernatant


14


15

from the same sources This time no abnormal cells grew It was concluded that no pathologic changes were induced in normal tissues by supernatant from gliomatous tissue throughout the observation period of 3 months providing adequate precautions were taken to exclude contamination by the original tumour cells

Exposure of Glioma Derived Cultures to RSV

The exposure of proliferating tumour derived cells to RSV (EH) was not followed by any demonstrable morphological change for the first month One month after virus exposure three cell lines 87 MG 105 MG and 118 MG developed small highly refractile oval to rounded areas composed of densely packed large mitosing cells These areas expanded slowly and formed multilayered heaps The transformation of an entire 50 mm plate took 4–6 weeks from overt onset Morphologically the appearance was highly reminiscent of Rous transformed human fibroblasts (*Stenkvist & Pontén* 1964) Transformed cells varied from the large giant cell whose cytoplasm contained vacuoles to spindle shaped or rounded forms with hyperchromatic polymorphic sometimes multilobed or multiple nuclei and a moderate amount of deeply basophilic cytoplasm (Fig 14)

Few S cells were present in the Rous transformed glial cultures The transformed cells were shed into the medium in large numbers (up to 400 000/dish) Maximal density of attached cells was usually about 180 000 cells/cm² Preliminary results from electron microscopic study of the RSV transformed glial cells indicate that they are different from transformed fibroblasts Repeatedly harvested supernatants failed to give any foci on highly susceptible monolayers of chicken fibroblasts No infectious RSV could thus be demonstrated

RSV transformed 118 MG-C cells caused six tumours in 16 inoculated chickens The tumours were histologically typical Rous sarcomas and

---

Figs 14–25

Fig 14 Line 118 MG C after transformation by RSV (FH) The field is dominated by multinucleated giant cells of typical Rous appearance Note the cell in the centre with mirror image symmetry and in the upper left corner the vacuolated giant cell with a central dense cytoplasmic area surrounded by numerous nuclei two cell types almost pathognomonic for transformation by RSV May Grunwald Giemsa Appr magn × 400

Fig 15 Boundary zone between normal glia (left and normal fibroblast like cells (right) The line 230 CC was obtained from gliosis rich in connective tissue surrounding a meningioma This line is not included in the present series but shows the unusual property of simultaneous growth of astrocyte and fibroblast like cells The latter grow at a higher density than the normal glia cells but can be distinguished from late passage MG-cells (see Fig 11) by their regular arrangement and growth in thin layers The pallissading of fibroblast nuclei which may be noted can be seen in fibroblastic cultures derived from most tissue May Grunwald Giemsa Appr magn × 90

two of these sampled for chromosome analysis showed chicken karyo types demonstrating that the neoplasms were induced in the recipients by material released from the inoculated cells.

DISCUSSION

Our present results indicate that glia cells may be the best material for the comparative study of normal and neoplastic human cells *in vitro*. Epithelium is not well suited for long term cultivation and carcinomas only rarely grow progressively (*Moore & Koike* 1964). Normal human mesenchyme survives well as long term cultures of fibroblast like cells which remain stable but neoplastic mesenchyme can only be carried in serial passage in about 5 per cent of all tumours (*Pontén & Saksela* 1967 and unpublished).

Normal glia shared essential features with normal human fibro blasts. The cultures were stable in the sense that no spontaneous trans formation into established or morphologically altered lines took place. The number of possible transfers which roughly would correlate with the maximal number of average cell doublings only varied from 10–17 (Table 2) and morphologically all control lines were indistinguishable and mainly composed of astrocytes. A regular monolayer arrangement was maintained suggesting the existence of 'contact inhibition' similar to that described for normal fibroblasts (*Abercrombie & Heaysman* 1954). Division decreased sharply at a well defined cell density indi cating that 'cell cycle inhibition' also described as a characteristic of normal fibroblasts (*Macieira Coelho, Pontén & Philipson* 1966) had not been lost.

Nineteen out of 30 malignant gliomas (63 per cent) were dominated by cytologically atypical cells capable of serial passage well beyond the control level. This rate of success in an unselected consecutive series is far above that obtained with any other type of human tumour. The clearest evidence of altered growth was a disturbed pattern with over lapping cells and an increased cell density suggesting at least a partial loss of 'contact inhibition of cell movement' and possibly also 'cell cycle inhibition'. In four instances (13.3 per cent) lines were formed which seemed capable of infinite propagation. The four tumours which developed into apparently permanently established lines (group C, Table 2) did not differ significantly from the tumours which gave rise to strains which had a finite life span *in vitro* (group B).

Ten out of 30 lines originating from gliomas showed no evidence of neoplastic growth. They resembled the control lines morphologically and were probably derived from stroma cells. Many of these biopsies came from necrotic or cystic grade III astrocytomas (Table 2). As regards three cases derived from grade I–II tumours it could not be determined whether highly differentiated glioma cells or stroma cells formed the lines.

The histogenetic ancestry of the abnormal cells of group B and C could not always be determined Structures staining like neurofibrils were however found at least in a proportion of the altered cells strongly suggesting that these cells were of astrocytic origin Cells with out fibrils often had an elongated shape somewhat resembling mesen chymal fibroblast like cells *in vitro* This was particularly prominent in line 105 MG-C 118 MG-C and 138 MG-C As to morphology and growth pattern these cells showed significant deviations from fibro blast like cells derived from normal human tissues and from glia rich in connective tissue removed from the vicinity of a meningioma (Fig 15) It therefore appears unlikely that they represented fibroblasts derived from normal vascular adventitia or arachnoid They may either represent mesenchymal cells which had undergone a sarcomatoid change or they may have represented non fibril forming variants of neoplastic glia cells Histologically the same difficulty is sometimes encountered and it has been proposed that malignant gliomas may contain a sarcomatous component (see *Willis* 1967)

The results of exposure to RSV are preliminary The absence of directly demonstrable infectivity and the production of sarcomas after inoculation into chickens parallel results with human fibroblasts trans formed by RSV (*Stenkvist & Ponten* 1964) Most likely the viral ge nome exists in the same masked state in both systems

SUMMARY

Three main findings resulted from the long term tissue culture of glial biopsies in humans First four glial cell lines capable apparently of indefinite proliferation *in vitro* were derived from the 30 gliomas successfully cultured Second immunoglobulin producing lymphoid cells grew out from one culture of a grade 3 astrocytoma rich in peri vascular lymphoid infiltration Third the *in vitro* life span of the nor mal glia and the remaining 25 gliomas was finite RSV (EH) induced a morphological transformation in the three glial lines tested and the transformed cells induced typical Rous sarcomata on injection into chickens

REFERENCES

1 *Abercombie M & Heaysham J E M* Social behavior of cells in tissue culture II Monolayering of fibroblasts Exp Cell Res *6* 93–306 1954

Ben jesh Melnick M Fernbach D J & Lewis R T Studies on human leukemia I Spontaneous lymphoblastoid transformation of fibroblastic bone marrow cultures derived from human leukemic and non leukemic chil dren J Nat Cancer Inst *31* 1311–25 1963

3 *Buckley R C* Tissue culture studies of the glioblastoma multiforme Amer J Path 5 467 472 1929

4 *Costero I & Pomerat C M* Cellular prototypes of central gliomata Proc 2nd Internat Congress of Neuropathology London pp 273–277 1955

5 *C x I B & C an we M I* Tissue culture of intracranial tumours J Path Bact 45 477–499 1937

6 *Fahey J J L Finegold I Rabson A S & Manaker R A* Immunoglobulin synthesis *in vitro* by established human cell lines Science *152* 1259–1261 1966
7 *Hayflick L & Moorhead P S* The serial cultivation of human diploid cell strains Exp Cell Res 25 585–621 1961
8 *Harrison R G* The outgrowth of the nerve fiber as a mode of protoplasmic movement J Exp Zool *9* 787–847 1910
9 *Jensen F C Girardi A J Gilden R V & Koprowski H K* Infection of human and simian tissue cultures with Rous sarcoma virus Proc Natl Acad. Sci U S *52* 53–59 1964
10 *Kredel F E* Tissue culture of intracranial tumours Amer J Path *4* 337–340 1928
11 *Lumsden C E In* Pathology of Tumours of the Nervous System D S Russell and L. J Rubinstein eds pp 143–149 1959
12 *Macieiro Coelho A Ponten J & Philipson I* The division cycle and RNA synthesis in diploid human cells at different passage levels *in vitro* Exp Cell Res *42* 673–684 1966
13 *Manuelidis E E & Pond A R* Continuous cultivation of cells arising from a human case of glioblastoma multiforme—Glioma TC 178 Proc Soc Exp Biol Med *102* 693–695 1959
14 *Marshall A H E* An outline of the Cytology and Pathology of the Reticular Tissue Oliver and Boyd. Edinburgh p 274 1956
15 *Moore C E & Koike A* Growth of human tumour cells *in vitro* and *in vivo* Cancer *17* 11–20 1964
16 *Peison B & Voris D* Primary sarcoma of the reticuloendothelial system of the brain Report of a case J Neurosurg 23 630–634 1965
17 *Penfield W & Cone W H In* McClung s Handbook of Microscopical Technique R McClung Jones ed. P B Hoeber New York. Ch 6 1950
18 *Pinkus H* The isolation of pure strains of cells from human tumors II Growth characteristics of a sarcoma and two brain tumors in tissue culture Amer J Cancer 29 25–46 1937
19 *Ponten J* Spontaneous lymphoblastoid in transformation of long term cell cultures from human malignant lymphoma Int. J Cancer 2 311–325 1967
20 *Ponten J & Saksela E* Two established *in vitro* cells lines from human mesenchymal tumours *Int J Cancer 2* 434–447 1967
21 *Ringert N* Grading of gliomas Acta path microbiol scandinav 27 51–64 1950
22 *Rubin H* The suppression of morphological alterations in cells infected with Rous sarcoma virus. J Nat Cancer Inst 9 141–159 1960
23 *Russell D S & Bland J O W* A study of gliomas by the method of tissue culture J Path Bact *36 273 283 1933*
24 *Saksela E & Moorhead P* Enhancement of secondary constrictions and the heterochromatic X in human cells Cytogenetics *1* 225–244 1962
25 *Samuelsson S M Werner I Ponten J Nathorst Windahl C & Thorell J* Reticuloendothelial (perivascular) sarcoma of the brain *Acta Neurol* scand 42 567–580 1966
26 *Shein H M* Propagation of human fetal spongioblasts and astrocytes in dispersed cell cultures Exp Cell Res *40* 554–569 1965
27 *Shein H M* Transformation of astrocytes and destruction of spongioblasts induced by a simian tumor virus (SV40) in cultures of human fetal neuroglia J Neuropath Exp Neurol 26 60–75 1967
28 *Stenkvist B & Ponten J* Morphological changes in bovine and human fibroblasts exposed to two strains of Rous sarcoma virus *in vitro* Acta path et microbiol scandinav *62 315–330 1964*
29 *Willis R A* Pathology of tumours 4th Ed Page 832 Butterworths London 1967

Acta path microbiol scandinav 74 487–494 1968

The Department of Pathology Bispebjerg Hospital, Copenhagen Denmark
(Head Charles Johansen)

PRIMARY SUPERFICIAL CARCINOMAS OF THE DUODENUM

By

ELLA LARSEN and AAGE JOHANSEN

Received 25 v 68

Our knowledge concerning early malignant changes in the small bowel is very restricted since tumours in this localization often are first considered late in the differential diagnosis of gastro-intestinal neoplasms This is the case with the duodenum too and especially with carcinomas of the first part of it In this region malignant tumours are exceedingly rare and since their symptoms are very like those displayed by diseases of the stomach or by duodenal ulcers the diagnosis is often first made when the process is widespread

It is the purpose of this paper to report two cases of initial malignancy in this the first part of the duodenum and to discuss the histogenesis Both cases are incidental findings in the duodenal edge of gastric ulcer resection specimens Since the very small carcinomas have hardly given any symptoms only a bried survey of the clinical histories will be given

CASE REPORTS

Case 1

Male patient born in 1884 Since 1926 he had suffered from pulmonary tuberculosis He had been treated with streptomycin and isoniazid Lately he had developed a fistula to the right axilla

He had never had any dyspepsia until December 1964 when he was admitted to this hospital with haematemesis Physical examination revealed an anaemic moderately ill old patient The blood pressure was 100/60 mm Hg., the haemoglobin was 5 9 g/per cent. The sedimentation rate 13 mm/h The bleeding continued and on January 25th 1965 a gastric resection according to the method Billroth I was performed (Dr D Andersen) The stoma h was full of blood and a superficial ulcer with a bleeding artery was found The duodenum was extraordinarily mobile and no infiltrations or ulcers could be palpated

The postoperative run was dominated by the senility of the patient He died three weeks after the operation

Pathological examination The gastric resection specimen revealed one cm distal to the proximal resectionborder a small ulcer The mucosa was moderately injected but without other focal processes At the distal part of the specimen a duodenal stump of about $2\frac{1}{2}$ cm was

seen This stump was macroscopically normal No lymph nodes were found

Microscopic examination revealed a small chronic ulcer situated in the top of the pyloric mucosa close to the small curvature The fundic mucosa showed a moderately atrophic chronic gastritis with severe reduction in the number of parietal cells In the pyloric mucosa a severe gastritis with extensive intestinal metaplasia was found No signs of malignancy were seen in relation to the ulcer or anywhere in the stomach

The duodenal stump was available for study in four out of sixteen examined sections At the first examination the only finding was a moderate inflammation but later at reexamination the following observations were made in one of the four sections The proximal 7 mm distal to the duodenal pyloric junction revealed nothing except a slight infiltration with lymphocytes and plasma cells in the lamina propria More distally the normal architecture of the mucosa was changed (Fig 1) The villi had disappeared and the crypts had lost their perpendicular arrangement and instead composed a trabecular network of anastomosing cords The cells were atypical without regular polarity The nuclei were enlarged hyperchromatic and a great number of mitoses including many tripolar and other atypical ones, were seen Transitions between normal crypts and atypical cords were seen Among the cords small clusters and also single tumour cells were seen in the lamina propria In the PAS stained sections some of the tumour cells showed a slightly positive reaction Nowhere did the malignant changes extend to the muscularis mucosae In the most proximal part of the malignantly changed area the mucosa was thin and had begun to ulcerate

In all sections from the duodenum small areas with a PAS positive gastric surface epithelium were seen intermingled with the duodenal epithelium

The Brunner glands had nearly completely disappeared under the malignantly changed epithelium About a centimeter of quite normal mucosa was seen distally In this part the Brunner glands reappeared and here small areas of metaplastic gastric epithelium were seen too In all the sections the serosa was normal

Autopsy A complete autopsy with microscopic examination was performed Only pertinent observations will be described The rest of the stomach was found to be normal The duodenum was without the slightest sign of malignancy The rest of the small bowel the colon the pancreas the bile ducts the liver and the lymph nodes were all without tumourformations Very severe tuberculous changes were found in the right lung and the right pleura *These together with severe atelectases were considered* the cause of death The cerebrum was normal *considering* the age of the patient

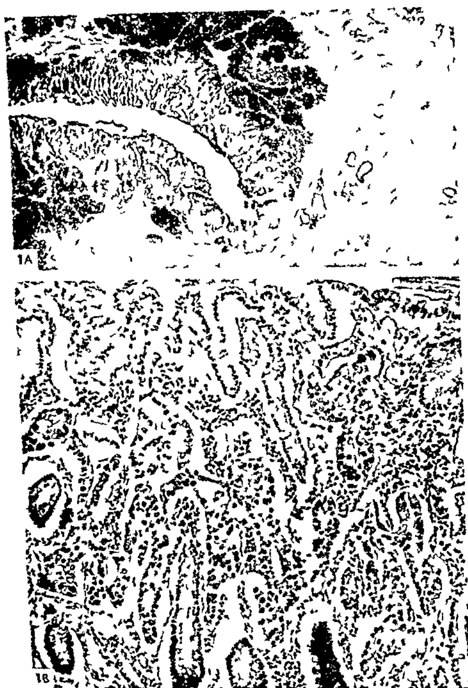


Fig. 1

Case 1. A. Survey. The superficial carcinoma occupies most of the [illegible] wn wards (distally). The brace indicate the positions of B (PAS [illegible]
B. Part of the carcinoma (H&E × 1[illegible]0)

Summary A 79 year-old man born in 1884 Since 1926 a diagnosis of pulmonary tuberculosis had been established After a few weeks with epigastric pains the patient developed severe gastric bleeding and Billroth I resection was made Three weeks later he died of pulmonary insufficiency Pathological examination of the gastric resection specimens revealed an ulcer situated between the pyloric and fundic mucosa No malignant changes were found in the stomach At microscopic examination of the duodenal stump at the distal part of the resection specimen a superficial carcinoma without any invasion of the muscularis mucosa was found 7 mm distal to the pylorus

The autopsy revealed no other malignant lesions

Case 2

This patient was a woman born in 1901 In 1941 she developed lymphadenopathia of the neck According to her own informations a malignant disease of uncertain nature had been diagnosed and she was given radiotherapy The enlarged lymph nodes disappeared and she has never had similar symptoms

In 1956 a cholecystectomy was performed From about this time she suffered periodically from dyspepsia and in 1963 and 1964 she had dietetic treatment in another hospital In February 1965 she was admitted to this hospital on account of increasing pains in the epigastrium vomitings and pyrosis Physical examination revealed a woman in good health The haemoglobin was 12 1 g/per cent Sedimentation rate 18 mm/per hour An augmented histamine test showed 76 meq/l and 11 8 meq/hour An X ray of the stomach showed a prepyloric ulcer On March 11th 1965 a Billroth II resection (Dr Bach Nielsen) was performed and a prepyloric ulcer was found The duodenum was normal on inspection and nothing abnormal could be palpated A frozen section of an enlarged lymph node in the ligamentum hepato duodenale showed no sign of malignancy The postoperative run was uncomplicated and the patient has been controlled in the out patient department several times latest May 1967 when she felt quite well

Pathological examination Macroscopic examination of the stomach revealed a small ulcer situated just above the pylorus It measured 9 by 12 mm and was without any macroscopic signs of malignancy The rest of the mucosa was normal Distal to the ulcer a duodenal stump 2 to 3 cm long was seen It was without any pathological changes No lymph nodes were found

Microscopic examination revealed a classical prepyloric ulcer on the small curvature At the distal border of the ulcer the very beginning of the duodenal mucosa was seen A moderately severe gastritis with a slight intestinal metaplasia was found in the pyloric part of the specimen In the fundic part of the specimen a superficial chronic gastritis was seen No sign of malignancy was disclosed in relation to the ulcer or anywhere else in the stomach

The duodenal stump could be studied in four sections In one of the sections (Fig 2) about half a centimeter above the distal resection border the following observations were made In the PAS stained section a collection of very heavily PAS positive cells were found in an extraordinarily large villus and also affecting the two neighbouring ones These cells were strongly Alcian blue positive too In the haem i



Fig 2

Case 2 A The whole extension of the carcinoma is illustrated (H&E × 110) B Section showing the signetring cells (H&E × 420) C The relation of the carcinoma to the Brunner glands (PAS × 20) D The strong Alcian blue reaction of the tumourcells (Alcian blue × 40)

toxylin eosin stained section it was seen that the cells in the most luminal part had the appearance of typical signetring cells. At deeper sites the cells were more anaplastic and contained only small drops of mucous. A few atypical mitosis were seen. The atypical cells invaded the stroma *between the crypts of Lieberkuhn but there* was *no* invasion of the Brunner gland layer and of the indistinct muscularis *mucosa. The surface epithelium covering the atypical cells showed some* irregular nuclei and had lost the goblet cells. The very first centimeter of the duodenum showed no pathological changes and neither did the centimeter nearest to the resectionborder. The Brunner glands were normal in all parts.

Small areas of metaplastic gastric surface epithelium were seen in this and the other sections from the duodenum. Apparently they had no relation to the malignant area. The serosa was normal.

Summary. A 64 year old woman born in 1901. According to her own information she had been treated for a malignant lymphadenopathia of the neck in 1941. This information could not be verified. Since 1956 she had experienced periods with gastric pains of ulcertype. After having received dietetic treatment several times, a Billroth II resection was performed in April 1965. The operation and postoperative run were uncomplicated. Three and a half years later she was doing well. Pathological examination of the gastric specimen revealed a prepyloric *ulcer. No malignancy was found in the stomach. Microscopical exami*nation of the duodenal stump disclosed a small superficial signetring *cell carcinoma* without any *invasion of the muscularis mucosa.*

DISCUSSION

Adenocarcinoma is by far the most common type of malignant neoplasms in the duodenum (*Machella* 1964). In case 1 the changes undoubtedly illustrate the beginning of an adenocarcinoma. The malignant changes in the crypts of Lieberkuhn and transitions from normal to atypical epithelium were seen in many places. The anastomosing cords and the presence of single tumour cells situated outside these cords indicated invasion of the lamina propria. Since no invasion of the muscularis mucosae had taken place, the correct designation of the changes, according to the similar conditions in the stomach, might be superficial or surface carcinoma of the duodenum. The same designation could be used in case *2*. Also here was an invasion of the lamina propria since the anaplastic signetring cells were disorderly arranged. Neither in this case was any invasion of the deeper part of the *mucosae*. Since nothing of the specimens were left, the exact size of the surface carcinomas could not be determined in either of the cases. The malignant changes were found in only one out of four sections from the duodenal stump, indicating their very limited size. Both

carcinoma is had a striking resemblance to the superficial carcinomas of the stomach described by *Konjetzny* (1940) *Morson* (1955) *Kuhlencordt* (1959) and others Since these two cases in fact are localized to the bowel mucosae the phenomenon may be a positive contribution to the discussion concerning the precancerous role of intestinal metaplasia in the stomach

On the other hand it was interesting to note a certain gastrification of the duodenal epithelium in both cases In case 1 corresponding to the malignant area all the villi and nearly all the Brunner glands had disappeared and small areas of gastric epithelium were seen in all sections from the duodenum The gastric epithelium was also seen in the duodenal sections from case 2 How often such metaplastic gastric epithelium is found in the duodenum is the subject of an as yet not finished investigation It is not a rare phenomenon It is not likely that the metaplastic epithelium has played an important role in the histogenesis of the two carcinomas but such metaplasia may have intensified their similarity with the gastric superficial carcinomas The possibility that the two carcinomas were not primary tumours can be left out of consideration The location in the most luminal part of the intestinal wall is a strong argument for this contention Furthermore the gastric specimens were part of a material investigated for early gastric cancer Both ulcers were completely sectioned and a total of sixteen sections from each specimens were examined but any signs of malignancy were not disclosed Besides the stomach carcinomas the malignant tumours of the pancreas are often seen to invade the first part of the duodenum In case 1 the autopsy revealed a normal pancreas and the history of case 2 precludes nearly with certainty a pancreatic carcinoma

To our knowledge such superficial carcinomas of the first part of the duodenum have never been reported All authors (*e g Dixon et al* 1946 *Vachella* 1964 *Wieners* 1966) agree that carcinomas in this position are extraordinarily rare Early but more extensive carcinomas in relation to duodenal ulcers have been reported (*Jefferson* 1916 *Dixon et al* 1946) It has been mentioned that malignant changes may take place in the Brunner glands and precancerous lesions have been reported by *Robertson* (1941) Early malignant transformations of heterotopic pancreatic tissue in this region (*Duff Foster & Bryan* 1943) as well as in the stomach (*Jarvi & Lauren* 1964) have been described but also these carcinomas have been more widespread

Some authors look upon the benign duodenal polyp as a precancerous lesion According to *Wieners* (1966) the adenomatous polyps are most common in the supraampullary part of the duodenum Malignant changes in such polyps have been reported by *Ebert et al* (1953) and by *Schiavi & Volta* (1964)

Although in this matter the starting point of duodenal carcinomas may be different most authors agree with the point of view that the

toxylin-eosin stained section it was seen that the cells in the most luminal part had the appearance of typical signetring cells. At deeper sites the cells were more anaplastic and contained only small drops of mucous. A few atypical mitosis were seen. The atypical cells invaded the stroma between the crypts of Lieberkühn but there was no invasion of the Brunner gland layer and of the indistinct muscularis mucosa. The surface epithelium covering the atypical cells showed some irregular nuclei and had lost the goblet cells. The very first centimeter of the duodenum showed no pathological changes and neither did the centimeter nearest to the resectionborder. The Brunner glands were normal in all parts.

Small areas of metaplastic gastric surface epithelium were seen in *this and the other sections from the duodenum.* Apparently they had no relation to the malignant area. The serosa was normal.

Summary. A 64 year old woman born in 1901. According to her own information she had been treated for a malignant lymphadenopathia of the neck in 1941. This information could not be verified. Since 1956 she had experienced periods with gastric pains of ulcertype. After having received dietetic treatment several times a Billroth II resection was performed in April 1965. The operation and postoperative run were uncomplicated. Three and a half years later she was doing well. Pathological examination of the gastric specimen revealed a prepyloric ulcer. No malignancy was found in the stomach. Microscopical examination of the duodenal stump disclosed a small superficial signetring cell carcinoma without any invasion of the muscularis mucosa.

DISCUSSION

Adenocarcinoma is by far the most common type of malignant neoplasms in the duodenum (*Machella* 1964). *In case 1* the changes undoubtedly illustrate the beginning of an adenocarcinoma. The malig*nant changes in the crypts of Lieberkühn and transitions from normal* to atypical epithelium were seen in many places. The anastomosing cords and the presence of single tumour cells situated outside these cords indicated invasion of the lamina propria. Since no invasion of the muscularis mucosae had taken place, the correct designation of the changes according to the similar conditions in the stomach might be superficial or surface carcinoma of the duodenum. The same designation could be used in case 2. Also here was an invasion of the lamina propria since the anaplastic signetring cells were disorderly *arranged.* Neither in this case was any invasion of the deeper part of the mucosae. Since nothing of the specimens were left, the exact size of the surface carcinomas could not be determined in either of the cases. The malignant changes were found in only one out of four sections from the duodenal stump, *indicating their very limited size.* Both

Acta path. microbiol. scandinav. 74: 495–513, 1968

The Institute of Pathology I, Karolinska Institutet, Stockholm

THE ULTRASTRUCTURE OF VIRUS AND RADIATION INDUCED THYMOMAS OF C57Bl MICE

By

BENGT LAGERLÖF

Received 2. v. 68

Iterated exposure to whole body irradiation induces thymomas in C57Bl mice. Cell free extracts of these thymomas have been shown to have leukaemogenic activity when inoculated intrathymically in newborn mice or into intrarenal thymic implants in thymectomized irradiated hosts (*Haran-Ghera et al.* 1966). These findings indicate the possibility of a virus being active in the leukaemogenic effect of irradiation. The first evidence of tumour following leukaemogenic treatment with irradiation or virus is crowding of immature blastic cells rich in mitotic figures in the cortex. Already at this very early stage of tumour development alkaline phosphatase appears in the tumour cells. This enzymatic activity does occur only in the reticular cells and endothelial cells of the vessels but not in the lymphoid cells of normal thymus (*Lagerlöf & Kaplan* 1967).

In attempts to further elucidate the nature of the radiation and virus induced thymomas an electronmicroscopical study of the tumours has been performed, initiated mainly by the two following questions:

1. Is the lymphoid character of the thymomas unequivocal? The very regular and early appearance of alkaline phosphatase in the tumour cells might indicate non-lymphoid origin of the neoplastic proliferation.
2. Can virus particles be demonstrated in radiation induced thymomas as well as in virus induced thymomas?

For this purpose radiation and virus induced thymomas have been examined in the electronmicroscope. For comparison with non-neoplastic thymus cells, thymus lobes from normal adult and embryonic mice were also examined. All material studied in the electronmicroscope was also examined in the light microscope and classified according to conventional histological criteria.

Supported by grants from *National Institutes of Health, U.S. Public Health Service* and the *Swedish Cancer Society*.

I should like to thank Dr. *Lydia Osvaldo-Decima* for valuable advice during the investigation.

Very efficient technical assistance by *Miss Maj Andersson* is gratefully acknowledged.

MATERIAL

Mice The mice were derived from the inbred strain C57Bl/Ka This strain has been maintained by continuous single line brother to sister mating The mice were kept on a standard pellet diet together with drinking water *ad libitum*

Induction of thymomas by irradiation treatment The mice were subjected to four doses of 170 r of unfiltered ^{60}Co irradiation separated by seven day intervals This treatment induces thymoma development in almost 100 per cent with latent period to death of 150–200 days (*Kaplan & Brown* 1952)

Induction of thymomas by virus treatment Weanling mice were thymectomized and exposed to four doses of whole body irradiation as indicated above One day after the last radiation exposure one thymic lobe from a newborn syngeneic donor of the same sex was implanted beneath the capsule of the left kidney One week later 0 02 ml of a virus preparation was injected directly into the thymic implant The virus preparation was obtained by differential centrifugation of leukaemic extracts from mice inoculated at birth with a passage of the virus (*Lieberman & Kaplan* 1959 *Haran Ghera et al* 1966)

Treatment of tissues for electronmicroscopy For electronmicroscopy the tissue was fixed in four per cent glutaraldehyde in cacodylate buffer at pH 7 2 for two hours and postfixed in one per cent osmic acid in veronal buffer at pH 7 2 for one hour The tissue was embedded in Epon and sections of about 400 Å thickness cut with a LKB Ultratome The sections were stained in 1 5 per cent uranyl acetate for one hour in 60 C and with lead acetate (*Millonig* 1961) and studied in a Siemens Elmiscope IA

Experimental material Thymus from ten adult normal mice and from twelve embryos aged 18 to 20 days together with eight radiation induced and six virus induced thymomas have been examined in the electronmicroscope

RESULTS

Adult Normal Thymus

Light Microscopy

The cells encountered histologically in the adult thymus are thymocytes epithelial cells and macrophages and occasional eosinophilic and basophilic granulocytes The thymocytes are densely packed in the cortex with single epithelial cells and macrophages interspersed The thymocytes are more scanty in the medulla which is made up of a loose reticulum of the epithelial cells containing scattered thymocytes The epithelial cells are abundant in the medulla but occur in a relatively smaller amount in the cortex Additional stromal support is rendered by the small vessels transversing the thymus and the rudimentary fibrous septa originating in the very thin fibrous capsule surrounding each thymus lobe

Electronmicroscopy

Survey pictures show the cortex populated by thymocytes with only occasional *epithelial cells and macrophages interspersed* (*Fig 1*) whereas the epithelial cells dominated the picture in the medulla (Fig 2)

Fixation artefacts such as rupture of mitochondria and nuclear membranes were present in some of the cells in the electron micrographs from normal as well as neoplastic thymuses Neither the nature nor

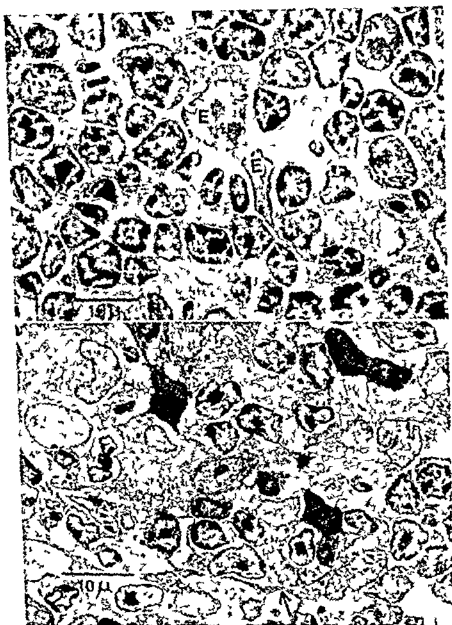


Fig. 1, 2

Fig. 1. Survey picture of thymus cort x from a normal adult m t . The maj rity of the cells are mature lymph i cells, the thymo yte . Tw ep th lial cell (E) are seen in the cent e f the fiel .

Fig. 2. Survey pi ture f thymus me lulla fr m a normal adult m u e. Several ep thelial c lls are seen. Th n in the lower part of the p t re h w el ar cytoplasmi extension ind cated by arrow.

the frequency of the artefacts appeared to affect the reproducibility of the results

Thymocytes Ultrastructurally the nucleus of the thymocyte is very regular oval or kidney shaped The nucleus shows distinct peripheral condensation of the chromatin which gives the cells definite lymphoid character also at the ultrastructural level Many of the thymocytes have one nucleolus located centrally in the nucleus The size of the nucleus in the electronmicrographs is usually 4–6 μ

The cytoplasm is scant and generally well circumscribed The cyto plasm contains a moderate amount of ribosomes a few oval or slightly elongated mitochondria (M) 0 3–0 7 μ in diameter and a small Golgi complex (G) (Fig 3) No desmosomes as described by *Lard* (1966) have been observed in the thymocytes

The thymocytes are regularly more electron dense than the other cells in the thymus and therefore easily recognized even at low magnifi cation

As a whole the ultrastructure of the thymocyte is very regular and the individual cells differ very little from each other

Epithelial cells The epithelial cells vary somewhat in appearance but have ultrastructural characteristics which make them easily re cognizable The nucleus is larger than that of the thymocytes round or oval and has a very finely dispersed chromatin containing one or two prominent nucleoli The cytoplasm is abundant and contains tono fibrils (T) These structures together with the desmosomes (D) con necting the cells give them their epithelial identity Moderate amounts of ribosomes are observed sometimes in polyribosomes Mitochondria are well developed measuring 0 3–1 0 μ in diameter and occur in moderate amount The Golgi complex is mostly well developed In some epithelial cells lysosomes measuring up to 0 5 μ in diameter are en countered Some of the epithelial cells also contain groups of cytoplas mic vacuoles (CV) varying in size but measuring up to 3 μ in diameter Amorphous material can often be seen in the vacuoles which are sur rounded by a membrane indented by microvillous projections (Fig 5)

The epithelial cells constitute a loose reticulum of the thymus with their long cytoplasmic extensions reaching into the intercellular spaces and connecting with other epithelial cells Very few empty intercellular spaces are encountered in the medulla and cortex of the adult thymus

---

Figs 3–4

Fig 3 Thymocyte from cortex from an adult thymus The peripheral clumping of the chromatin is clearly evident The Golgi complex (G) is seen in the right upper part of the cell There is a moderate amount of free ribosomes

Fig 4 Epithelial cell from medulla of a normal adult thymus In the cytoplasmic extension tonofibrils (T) and desmosomes (D) are seen


G
3
1μ
D
4
1μ

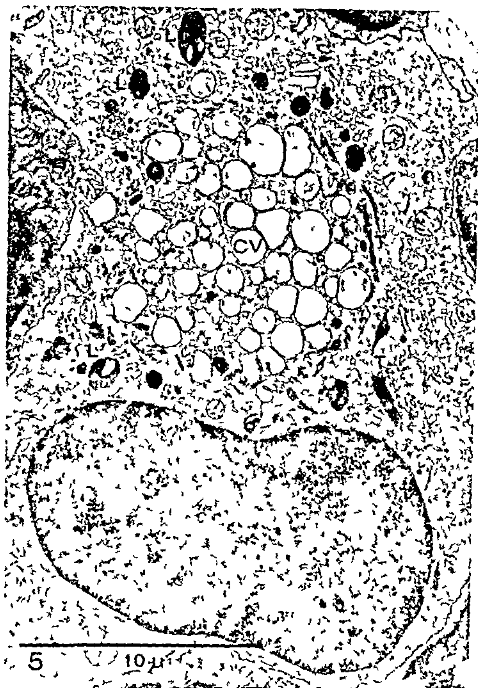


Fig 5

Epithelial cell from cortex from a normal adult thymus A large group of cytoplasmic vacuoles is seen (CV) and tonofibrils (T) and lysosomes (L) are evident



Fig 6

Macrophage from medulla of a normal adult thymus The cell contains a prominent rough surfaced endoplasmic reticulum (ER) and several large lysosomes (L)

since they are mostly occupied by these cytoplasmic extensions of the epithelial cells

Immature mesenchymal cells and macrophages These cells occur both in the cortex and the medulla though to a lesser extent than the epithelial cells They have large oval nuclei with finely dispersed chromatin sometimes containing one or two nucleoli The cytoplasm is usually abundant well circumscribed and devoid of cytoplasmic processes Desmosomes and tonofibrils do not occur The ribosomes are

often attached to the endoplasmic reticulum but can also occur free in the cytoplasm (Fig 6) Mitochondria measuring 0 3–1 0 μ in diameter occur in moderate amount The Golgi complex is not prominent Groups of lysosomes are usually encountered measuring up to 1 5 μ in diameter Small empty vacuoles can sometimes be seen in the cytoplasm

Additional cell components In addition to the above mentioned main cell types occasional eosinophilic and basophilic granulocytes have been observed These cells are easily recognized because of their characteristic granulation

No virus particles have been observed in the thymus of normal untreated adult mice

The ultrastructural morphology of the cells in the normal thymus from C57Bl mouse agree principally with the findings in strain 129/J mouse reported by *Clark* (1963) and in sprague Dawley rats reported by *Lundin & Schelin* (1965)

Embryonal Normal Thymus

Light Microscopy

Histologically the thymus from embryos aged eighteen to twenty days is not separated into a medulla and a cortex as the adult thymus but the whole organ is populated by immature blast like cells and some more mature lymphoid cells laying in a loose reticulum of epithelial cells

Electronmicroscopy

At the electronmicroscopical level the cells can be identified as follows

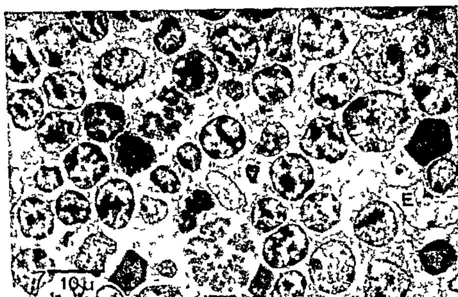
Immature, lymphoid cells lymphoblasts The majority of the cells in the thymus of embryos aged eighteen to twenty days are immature lymphoid cells with uniform ultrastructural morphology (Fig 7) The nucleus is round or oval measuring 5–7 μ in diameter (Fig 8) The chromatin is finely dispersed in the centre but usually clumped at the periphery though not to the same extent as in the mature lymphoid

---

Figs 7–8

Fig 7 Survey picture of thymus from mouse embryo The majority of the cells are immature lymphoid cells lymphoblasts some of which are in the process of mitotic division Occasional epithelial cells (E) are also seen

Fig 8 Lymphoblast from thymus from mouse embryo The chromatin does not show pronounced peripheral clumping at the nuclear membrane (compare with Fig 3) Moderate amounts of ribosomes are seen in the cytoplasm partly as polyribosomes


E
7
10μ

cell One or two nucleoli can often be seen The cytoplasm is round and can show small microvilli but no cytoplasmic extensions Ribo somes occur in large amounts often as polyribosomes and are much more abundant than in the fully mature thymocyte Mitochondria occur in moderate amounts as in the mature thymocytes and are often small measuring 0 2–0 6 μ in diameter The Golgi complex is usually not prominent

More differentiated lymphoid cells The embryonic thymus also contains lymphoid cells which are more mature and differentiated than the lymphoblasts All degrees of differentiation up to the fully mature thymocyte can be encountered The stage of differentiation has been judged by the degree of peripheral condensation of the chromatin which is most pronounced in the mature cells and the amount of ribo somes which decreases with increasing differentiation The number of immature cells has been in majority in all embryonic thymus lobes and the more or less mature lymphoid cells have been seen scattered among the lymphoblasts

Epithelial cells The epithelial cells constitute the reticulum of the embryonic thymus as in the adult thymus They occur in the same amount as in the cortex of adult thymus Pictures similar to the loose medulla of the adult thymus where epithelial cells constitute a greater part of the cell populations are not seen The ultrastructure of the embryonic epithelial cells is the same as in the adult thymus

Immature mesenchymal cells and macrophages Immature mesen chymal cells and macrophages with the same ultrastructural picture as in the adult thymus are seen also in the embryonic thymus

No virus particles like those described by *Carnes* (1967) have been observed in the embryonic thymus lobes

Thymomas

Light Microscopy

The tumours present generally a monomorphic histological picture The cell population is dominated by the neoplastic lymphatic cells laying densely packed together and very few other structures are en countered Some small vessels are seen and occasional macrophages and epithelial cells can be observed in some tumours No structures are seen to constitute a stroma in the thymomas similar to the loose epithelial reticular stroma in the normal thymus A very thin fibrous capsule infiltrated by tumour cells surrounds the tumour tissue

The tumours have definite lymphoid character histologically with round or oval nuclei containing one or two nucleoli and more or less pronounced peripheral clumping of the chromatin The cytoplasm is usually scant and well circumscribed Only in the very low differen tiated tumours a more pronounced pleomorphism is evident and the lymphoid appearance of the single tumour cells lost



Fig 9

Tumour cell from a moderately differentiated virus induced thymoma The nucleus shows slight peripheral condensation of the chromatin The inserted field shows virus particles in the process of budding off from the cell membrane

Electronmicroscopy

Also at the ultrastructural level the thymomas are usually very uniform

The differences occurring can be correlated mainly with differences in the degree of differentiation Therefore the thymomas have been grouped together according to their degree of differentiation and the ultrastructure of the tumour cells of two groups encountered for

cell One or two nucleoli can often be seen The cytoplasm is round and can show small microvilli but no cytoplasmic extensions Ribosomes occur in large amounts often as polyribosomes and are much more abundant than in the fully mature thymocyte Mitochondria occur in moderate amounts as in the mature thymocytes and are often small measuring 0 2–0 6 μ in diameter The Golgi complex is usually not prominent

More differentiated lymphoid cells The embryonic thymus also contains lymphoid cells which are more mature and differentiated than the lymphoblasts All degrees of differentiation up to the fully mature thymocyte can be encountered The stage of differentiation has been judged by the degree of peripheral condensation of the chromatin which is most pronounced in the mature cells and the amount of ribosomes which decreases with increasing differentiation The number of immature cells has been in majority in all embryonic thymus lobes and the more or less mature lymphoid cells have been seen scattered among the lymphoblasts

Epithelial cells The epithelial cells constitute the reticulum of the embryonic thymus as in the adult thymus They occur in the same amount as in the cortex of adult thymus Pictures similar to the loose medulla of the adult thymus where epithelial cells constitute a greater part of the cell populations are not seen The ultrastructure of the embryonic epithelial cells is the same as in the adult thymus

Immature mesenchymal cells and macrophages Immature mesenchymal cells and macrophages with the same ultrastructural picture as in the adult thymus are seen also in the embryonic thymus

No virus particles like those described by *Carnes* (1967) have been observed in the embryonic thymus lobes

Thymomas

Light Microscopy

The tumours present generally a monomorphic histological picture *The cell population is dominated by the neoplastic lymphatic cells* laying densely packed together and very few other structures are encountered Some small vessels are seen and occasional macrophages and epithelial cells can be observed in some tumours No structures are seen to constitute a stroma in the thymomas similar to the loose epithelial reticular stroma in the normal thymus A very thin fibrous capsule infiltrated by tumour cells surrounds the tumour tissue

The tumours have definite lymphoid character histologically with round or oval nuclei containing one or two nucleoli and more or less pronounced peripheral clumping of the chromatin The cytoplasm is usually scant and well circumscribed Only in the very low differentiated tumours a more pronounced pleomorphism is evident and the lymphoid appearance of the single tumour cells lost



Fig 12

Tumour cell from the radiation induced thymoma shown in Fig 11 The nucleus shows characteristic peripheral condensation of the chromatin and one prominent nucleolus The inserted field shows several virus particles in the cytoplasm and perinuclear cisterne

circumscribed Large amounts of ribosomes and polyribosomes are seen in these cells Mitochondria mostly occur in moder[ate] amounts but some cells can show up to ten mitochondria in one section They are usually small or moderately sized usually measuring 3[–]5 µ in diameter The Golgi complex is not prominent The endoplasmic reti culum is sparse but is in some places distended forming small vesicles In some cells occasional lysosomes are observed

In attempts to discover the occurrence of virus particles in normal untreated mice Carnes was able to identify C particles in embryonic thymus In the present study, no virus particles were observed in non leukaemic thymus lobes either from adult or embryo in spite of very careful search for virus particles

SUMMARY

Thymus lobes from normal adult C57Bl mice and embryos and thymomas induced by radiation and virus have been studied in the electron microscope In the normal adult thymus thymocytes epithelial cells immature mesenchymal cells and macrophages are encountered Occasional eosinophilic and basophilic granulocytes are also seen The thymocytes have distinct peripheral clumping of the chromatin giving the cell the characteristic lymphoid appearance This was evident also in the lymphoblasts from the embryos though the lymphoblasts did not show such a distinct and pronounced condensation of the chromatin The epithelial cells which make up the loose reticulum of the stroma of the thymus were characterized by the presence of tonofibrils and desmosomes and occasional lysosomes The immature mesenchymal cells and macrophages often exhibited a well developed rough endoplasmic reticulum in addition to small groups of lysosomes The ultrastructure of the tumour cells was generally very regular and similar to that of lymphoblasts from normal embryos Occasional lysosomes could be observed in some tumour cells The amount of ribosomes was larger in lymphoblasts and tumour cells than in normal thymocytes from adult mice Histological classification into high and low differentiated tumours was in the electromicrographs seen to correspond to less pronounced peripheral condensation of the chromatin in the nucleus of the low differentiated tumour cells whereas no particular differences were evident in the cytoplasm In one out of a total of six virus induced thymomas virus particles of C type were observed in the cellular spaces and in the process of budding off from the cell membrane In one radiation induced thymoma similar particles were observed intercellularily and in the cytoplasm

REFERENCES

Ackerman G A & Knouff R A The epithelial origin of the lymphocytes in the thymus of the embryonic hamster *Anat Rec 152* 35 54 1965

Bernhard W & Granboulan N Morphology of oncogenic and non oncogenic mouse viruses A Ciba Foundation Symposium on Tumour Viruses of Murine Origin Ed Wolstenholme and O Connor 1962

Carnes W H Radiation leukemia virus in embryonic C57Bl mouse thymus Fed Proc 26 748 2745 1967

Clark S L The thymus in mice of strain 129/J studied with the electronmicroscope Amer J Anat *112* 1-34 1963

Dalton A J Law L W Moloney J B & Manaker R A An electronmicroscopic study of a series of murine lymphoid neoplasms J Nat Cancer Inst 27 747-791 1961

de Harven F & *Friend C* Further electronmicroscope studies of a mouse leukemia induced by cell free filtrates J Biophys Biochem Cytol 7 747-75? 1960
Haran Ghera N Leukemogenic activity of centrifugates from irradiated mouse thymus and bone marrow Int J Cancer 1 81-87 1966
Haran Ghera N *Lieberman M* & *Kaplan H S* Direct action of a leukemogenic virus on the thymus Cancer Res 26 438-44? 1966
Karl J Presence de desmosomes entre des cellules épithéliales et des cellules lymphocytaire dans le thymus de cobaye J Microscopie 5 3 1-3?4 1966
Kaplan H S On the natural history of the murine leukemias. Cancer Res. 27 13?5-1340 1967
Kaplan H S & *Brown M B* A quantitative dose response study of lymphoid tumor development in irradiated C57 black mice J Nat Cancer Inst 13 185-208 195?
Lagerlof B A M & *Kaplan H S* Specificity of the relationship between thymic alkaline phosphatase activity and lymphoma development in strain C57Bl mice J Nat. Cancer Inst 38 437-458 1967
Lieberman M & *Kaplan H S* Leukemogenic activity of filtrates from radiation induced lymphoid tumors of mice Science 130 387-389 1959
Millonig G Modified procedure for lead staining of thin sections J Biophys Biochem Cytol 11 736 1961
Lundin P M *Schelin U* Ultrastructure of the rat thymus Acta path microbiol scandinav 65 379-394 1965

Acta path microbiol scandinav 74 514-518 1968

Department of Forensic Medicine (Head Prof T Saldeen) Institute of Pathology University of Uppsala Uppsala Sweden

CORTISONE NEPHROPATHY IN THE RABBIT

The Effect of Warfarin Administration

By

LENNART RAMMER

Received 14 x 68

Daily injection of corticosteroids in rabbits gives rise after about two weeks to well defined homogeneous lesions in the renal glomeruli (*Rich et al* 1950 *Rosen et al* 1954 *Bloodworth & Hamwi* 1955 *Wilens & Stumpf* 1955 *Moran et al* 1962 *Wilson et al* 1962 *Ogilvie et al* 1965 *Rammer et al* 1967) It has been shown by electrone microscopy that these lesions are localized mainly within the lumina of the capillaries and that they contain red blood cells (*Moran et al* 1962 *Ogilvie et al* 1965) Immunohistochemical methods have revealed that the material contains plasma constituents such as albumin globulin and fibrinogen On the basis of these morphological studies it has been suggested that *the cortisone induced renal changes in the rabbit may* comprise a form of capillary thrombi (*Moran et al* 1962) Conclusive proof of this hypothesis is however still lacking

In a previous investigation (*Rammer et al* 1967) considerable potentiation of the cortisone induced renal changes was obtained by means of simultaneous daily administration of heparin This effect of heparin seemed difficult to understand if these lesions did actually comprise thrombi In order to elucidate further the importance of the coagulation process for the development of the cortisone induced renal changes in the rabbit the effect of another anticoagulant warfarin was therefore studied

MATERIAL AND METHODS

Cortisone 7 5 mg of cortisone acetate as a 25 per cent aqueous suspension (Cortal® Pharmacia) was injected intramuscularly once daily

Warfarin Warfarin sodium (Waran® Nyegaard) was injected intramuscularly once daily The dose required to obtain a thrombotest level (see below) of between 10 and 20 per cent usually lay between 1 and 5 mg daily As a control of the anticoagulant therapy *Thrombotest®* (Nyegaard) was used This test is sensitive to depression of the blood coagulation factors in the prothrombin complex (factors II VII IX and X) which are affected by dicoumarol therapy For the thrombotest *determinations blood from the marginal* vein of one ear was used and analyses were made almost daily

Antibiotics As a protection against infections all animals were given daily intramuscular injections of 0 15 ml of Streptopenin® (KABI) containing 200 000 IU of benzylpenicillin procaine and 0 25 g of streptomycin sulphate per ml

Animals 32 male albino rabbits weighing between 2.2 and 2.6 kg were used. They were given 130 g of Ewos pellets daily and free access to water. After 21 days of injections the animals were killed by an overdose of Pentothal sodium® (Abbott) given intravenously.

Morphological Methods

Autopsy was performed immediately, the kidneys and livers were weighed and specimens were fixed in 10 per cent neutral formalin. Frozen sections were stained with Scharlach Rot and Sudan Black. Paraffin embedded sections were stained with eosin haematoxylin, periodic acid Schiff (PAS), phosphotungstic acid haematoxylin (PTAH), Wilder's reticulin stain and Congo Red. For the quantitative analyses of the hyaline lesions in the renal glomeruli a PAS stained 5 μ thick cross section from the central region of one kidney from each animal was used. Glomeruli containing hyaline material were counted and expressed in per cent of the total number of counted glomeruli in the section (approximately 400–800).

RESULTS

The animals were divided into four groups (Table 1). During the course of the experiment one animal treated with cortisone alone died of pulmonary infarction, two animals treated with both cortisone and warfarin and one animal treated with warfarin alone died of haemorrhage from the sites of venipuncture. Thus, at the end of the experiment there remained 11 cortisone treated animals, 10 treated with cortisone and warfarin and 2 treated with warfarin, and also 4 untreated controls. Only these animals will be reported on below.

TABLE 1

The Percentages of Glomeruli Containing Hyaline Lesions and the Weights of Kidney and Liver in the Four Experimental Groups. Mean ± s.e.m.

Group	Animals	Per cent glomeruli with lesions	Kidney weight g/kg bodyweight	Liver weight g/kg bodyweight
Cortal	11	2.3 ± 0.7	8.06 ± 0.42	71.9 ± 3.3
Cortal Waran	10	0.3 ± 0.1	8.55 ± 0.40	71.7 ± 4.0
Waran	2	0	6.28 ± 0.1	35.0 ± 1.1
Controls	4	0	5.33 ± 0.37	24.53 ± 0.74

The kidneys in the two cortisone treated groups were oedematous and enlarged (Table 1). The weight of the kidneys per kg body weight was 8.06 ± 0.42 g in the cortisone group, 8.55 ± 0.40 g in the cortisone warfarin group and 6.28 ± 0.15 g in the warfarin group compared with 5.33 ± 0.37 g in the control group.

On microscopic examination of the kidneys well defined hyaline lesions of the type described previously (*Rammer et al.* 1967) were seen in the cortisone treated animals (Fig. 1). Quantitative analysis of the renal changes in the cortisone treated animals showed that 2.3 ± 0.7 per cent of the glomeruli exhibited hyaline lesions, while in the animals treated with both cortisone and warfarin the corresponding figure was 0.3 ± 0.1 per cent. This difference is statistically significant ($p < 0.001$).

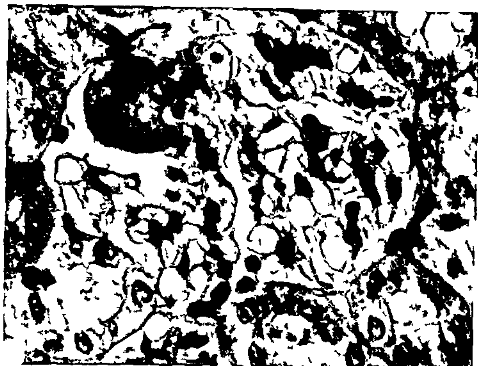


Fig 1

Glomerulus of a cortisone treated rabbit containing a rounded homogenous lesion PAS

The livers in the two cortisone treated groups were significantly enlarged (Table 1) and showed considerable macroscopic and micro scopic fatty deposits The liver weight per kg body weight was 73 9 ± 3 3 g in the cortisone group 71 7 ± 4 0 g in the cortisone warfarin group and 35 0 ± 1 1 g in the warfarin group compared with 24 53 ± 0 74 g in the controls

DISCUSSION

The fact that the renal lesions were inhibited in the present investi gation by the administration of warfarin simultaneous with the corti *sone must mean that coagulation is necessary for their development* and thus strengthens the assumption that they consist of thrombi

The results of our previous investigation (*Rammer et al* 1967) in which considerable potentiation of the renal changes was obtained by means of simultaneous administration of heparin however seem diffi cult to interpret *if this view is to be accepted*

The paradoxical potentiating effect of heparin on the renal changes might however be explained as follows The cortisone therapy gives rise to increased mobilization of free fatty acids (FFA) from adipose tissue which fact is manifested in the form of loss of weight fatty

infiltration of the liver and extreme lipidemia *Connor et al* (1963) found that injection of FFA in the rabbit gave rise to extensive thrombosis and *Hoak et al* (1963) obtained the same result after endogenous mobilization of FFA by injection of ACTH The potentiating effect of heparin on the formation of thrombi could then be due to the fact that heparin liberates the enzyme lipoprotein lipase (clearing factor) to plasma resulting in hydrolysis of the large quantity of circulating triglycerides and a further increase in the plasma FFA concentration The potentiating effect of heparin on the coagulation would thereby predominate over its coagulation inhibiting action

Several findings indicate however that further factors must also be involved in the pathogenesis of cortisone nephropathy in the rabbit The cortisone induced renal lesions do not have the same appearance as in other types of intracapillary fibrin deposits in the renal glomeruli for example in the generalized Shwartzman reaction or after injection of thrombin The cortisone induced lesions lack the typical parallel arrangement of the fibres seen in these other conditions and have instead an amorphous homogeneous character The blue tone in PTAH staining is rather lighter than in ordinary fibrin thrombi In addition the cortisone induced lesions are rounded and completely fill greatly dilated capillaries while the other types of thrombi fill out capillaries of normal width Another dissimilarity lies in the time of manifestation and disappearance of the changes On injection of thrombin thromboplastin or FFA for example an extensive intracapillary precipitation of fibrin is seen within a few minutes and this fibrin disappears within about one hour (*Saldeen* 1966) The cortisone induced renal changes in the rabbit do not appear until after about 10–14 days therapy and remain for a long period even after the cortisone treatment has been discontinued (*Bloodworth & Hamwi* 1955) The renal lesions induced by cortisone in the rabbit thus differ from other forms of intravascular coagulation in the renal glomeruli both as regards their morphology and their course of development

One reason why the changes disappear so slowly and perhaps also why there is a dissimilarity in appearance compared with other types of thrombi may be that they are a result not only of an intravascular coagulation but also of a reduced capacity for removal of the fibrin Two mechanisms for such a reduced capacity may exist Firstly *Vaselli & McCluskey* (1963) showed that the endothelial cells in the glomeruli in the rabbit had the capacity of phagocytizing intravascularly deposited fibrin and other particulate material and they thus comprise a part of the reticulo-endothelial system (RES) After blockade of this system with for example thorotrast a single dose of endotoxin or thrombin induced extensive thrombosis as in the generalized Shwartzman reaction (*Lee* 1962) The same type of extensive thrombosis after a single dose of *endotoxin was obtained* when rabbits had been premedicated with cortisone (*Thomas & Good* 1952) Since it is known that

cortisone also gives rise to RES blockade it is possible that changes in the reticuloendothelial system may be of importance for the development of cortisone nephropathy in the rabbit

Secondly the delayed disappearance of fibrin in cortisone therapy could be a disorder of the fibrinolytic system Too little is known as yet however about the condition of this system either in cortisone therapy or in intravascular coagulation for any assessment of this possibility to be made

SUMMARY

Rabbits treated with cortisone for 3 weeks developed rounded homogenous hyaline lesions in the renal glomeruli The development of this cortisone nephropathy in the rabbit could be inhibited to a major extent by simultaneous administration of warfarin indicating that these changes are dependent upon a coagulation process The importance of FFA mobilization and disturbances in the reticulo endothelial and fibrinolytic systems in this connection is discussed

REFERENCES

1 *Bloodworth J M B & Hamwi G J* Histopathology of experimental glomerular lesions simulating human diabetic glomerulosclerosis Am J Path 31 167–187 1955
2 *Connor W E Hoak J C & Warner E D* Massive thrombosis produced by fatty acid infusion J Clin Invest 42 860–866 1963
3 *Hoak J C Poole J C F & Robinsson Ph D* Thrombosis associated with mobilization of fatty acids Am J Path 43 987–995 1963
4 *Lee L* Reticuloendothelial clearance of circulating fibrin in the pathogenesis of the generalized Shwartzman reaction J Exp Med 115 10 5–1082 1962
5 *Moran T J Kurt S M & Vazquez J J* Diabetic and cortisone induced renal lesions A morphological and immunohistochemical study Lab Invest 11 240–254 1962
6 *Ogilvie R F Sabour M S & Horne N W* Light and electrone microscopy of prednisolone induced nephropathy in rabbits Diabetes 14 595–605 1965
7 *Rammer L Nathorst Windahl G & Boberg J* Cortisone nephropathy in the rabbit. The effect of heparin on the morphology plasma lipid levels and plasma lipoprotein lipase activity Acta path microbiol scandinav 71 161–172 1967
8 *Rich A R Berthrong M & Bennet I I* The effect of cortisone upon the experimental cardiovascular renal lesions produced by anaphylactic hypersensitivity Bull John Hopkins Hosp 87 549 568 1950
9 *Rosen D A Beller B Mangwyn Davies C D & Friedenwald J S* Influence of heparin on cortisone nephropathy of the rabbit Bull John Hopkins Hosp 95 144–146 1954
10 *Salleen T* Experimental investigation on fat embolism and intravascular coagulation Acta path microbiol scandinav 66 271 1966
11 *Thomas L & Good R A* The effect of cortisone on the Shwartzman reaction J Exp Med 95 409 427 1952
12 *Vaselli P & McCluskey R T* The pathogenetic role of the coagulation process in rabbit Masugi nephritis Am J Path 45 653–677 1964
13 *Wilens S L & Stumpf H H* Nodular and fatty glomerular lesions in rabbits on cortisone Am J Path 31 275 295 1955
14 *Wilson D I Rosen D A & Bencosme S A* Steroid nephropathy in the rabbit Diabetes 11 402–411 1962

Acta path microbiol scandinav 74 519–530 1968

The Blood Center and the Department of Clinical Chemistry University Hospital
Uppsala Sweden

QUANTITATIVE IMMUNOGLOBULIN DETERMINATION

Comparison of two Methods Estimation of Normal Levels and Levels in Persons Lacking IgA and IgD

By

S G O Johansson Claes F Hogman and Johan Killander

Received 23 ii 68

Separate determination of the immunoglobulins G A M and D is of great value for different purposes e g in the diagnostics of hypogammaglobulinaemia and myeloma/macroglobulinaemia Sometimes a high degree of sensitivity is required of the method e g when antibody eluates or other fractions with extremely low concentration of the respective immunoglobulin are to be tested In other cases a too high degree of sensitivity is only cumbersome and results in less accuracy

The aim of the present paper is to report on some experiences gained with a highly sensitive technique the passive haemagglutination inhibition technique according to *Boyden* (5) as modified by *Wide* (32) and also with a less sensitive method the single radial immunodiffusion method of *Mancini et al* (23)[1] Furthermore the frequency at which lack of IgA and IgD in serum is encountered in a population is reported The concentration of the other immunoglobulins in sera from the IgA agammaglobulinaemia patients has been investigated

MATERIAL AND METHODS

Sera from 89 individuals between the ages of 20 and 70 and with an *even sex and* age distribution were taken to represent a normal Swedish population They were found to be healthy according to the ESR haemoglobin concentration urinary

The authors wish to express their gratitude to Dr *Lars Linder* who kindly supplied the blood samples from an examination of healthy people to Dr *Lars Beckman* who provided the samples from homozygotic and heterozygotic twins to Dr *D S Rowe* for antisera and reference preparations and to Dr *Renée Norberg* for reference preparations This work was supported by grants from the *Swedish Medical Research Council* (B68–16X–105–04) and *the Faculty of Medicine University of Uppsala* which are gratefully acknowledged

[1] *The following abbreviations are used*
PHI = Passive haemagglutination inhibition technique
SRD = Single radial diffusion technique
PBS = Phosphate buffered saline one part of 1/15 M Soerensen phosphate buffer pH 6 4 and four parts of 0 14 M NaCl solution
NRS = Normal rabbit serum

protein and sugar test and on physical examination All the sera were analysed by immunoelectrophoresis against a polyvalent immunoglobin antiserum One serum contained an M-component of the IgG type and was not included in the calculations Moreover sera from 3937 blood donors and 1401 pregnant women were investigated Finally 3 patients who showed no signs of haematological disorders but lacked IgA were tested

Immunoglobulin Preparations

A purified preparation of IgG was obtained through the courtesy of AB Kabi (Stockholm) It was prepared by Cohn fractionation and DEAE chromatography and was stored as a 12 per cent solution in 0 3 M glycine For use as a reference standard the preparation was further purified by gel filtration on Sephadex G 200

A purified preparation of IgA from normal sera was kindly provided by Behringwerke AG (Marburg/Lahn Germany) IgA was also prepared from two myelomatous sera by gel filtration and electrophoresis (21)

IgM was isolated by gel filtration and electrophoresis (17) from pooled normal serum and the serum of a patient with macroglobulinaemia Another IgM preparation was obtained from a cryomacroglobulinaemia serum by repeated precipitation and dissolving

IgD was isolated from a myelomatous serum by starch block electrophoresis and gel filtration on Sephadex G 200

The immunoglobulin preparations were kept at —20 C. The purified products showed no impurities or cross reacting material (less than 5 per cent) when tested by immunoelectrophoresis and agar gel diffusion techniques with antisera against normal human serum and antiserum specific for the different immunoglobulins

Most of the antisera used were prepared by the immunization of rabbits with immunoglobulin preparations described above (16) Some sera were obtained from Behringwerke AG To render the antisera specific absorptions were made for *anti IgG* with the two IgA myeloma proteins and an IgM preparation from normal serum for *anti IgA* with serum from a healthy person lacking IgA (L.S) for *anti IgM* with IgG globulin and a cord serum with low IgM content for *anti IgD* with serum from a healthy subject lacking IgD A specific *anti IgD* serum (N84) was obtained through the courtesy of Dr D S Rowe

Fractionation of serum proteins by gel filtration and preparative electrophoresis was carried out as previously described (21) The protein concentration in diluted samples was increased by ultrafiltration in collodion bags Protein concentrations were determined as described previously (16) by the use of a modified Folin method or by absorbancy at 280 nm using as standard 14 0 for a 1 per cent solution in 0 01 M hydrochloric acid and by Kjeldahl nitrogen analyses Immunoelectrophoresis and double diffusion in agar gel were carried out as described (16)

Single Radial Diffusion Technique (SRD) was applied according to Mancini et al (23) with some modifications One part of 3 per cent agar (Special Agar Noble Difco) in 0 3 M phosphate buffer pH 8 0 was mixed with one part of antiserum usually diluted 1 20–1 40 in 0 14 M NaCl In the agar antiserum layer (12 × 12 × 1 mm) 2 0 mm holes were cut and filled once with 2 µl and for IgD determination twice with 2 5 µl of a suitable dilution of each sample and standard The diameters of the unstained precipitin zones were determined using a measuring magnifier after incubation at room temperature for 18–24 hours for IgG IgA and IgD and for 48 hours for IgM A series of four dilutions of a standard serum and a control serum were applied in duplicate to each plate The concentration of the different immunoglobulins in the standard serum was separately determined using purified IgG IgA IgM and IgD preparations as standards The area of the precipitin zones for the standards were plotted on a linear scale against the concentration A straight line was obtained Each sample was run in duplicate If the diameter between duplicate samples exceeded 0 2 mm the result was discarded

The Passive Haemagglutination Inhibition Technique (PHI) according to Boyden (5) was used with the modifications described by Wide (32) To a dilution of the respective anti Ig serum red cells coated with antigen were added The agglutination thus obtained was inhibited by standards and samples diluted by serial titration By comparing the end point agglutination pattern with that of a standard the concentration of immunoglobulin in the sample could be calculated Details of the technique are given below A brief report was given in (19)

Preparation of red cells for the PHI test An 8 per cent suspension of washed human red blood cells blood group O Rh negative was collected in Alsever's solution and mixed with an equal volume of a 1 per cent solution of formaldehyde in saline adjusted to pH 7.0 (29). The mixture was incubated for 12 to 18 hours at 37 C. After washing four times with distilled water the cells were stored at 4 C as a 20 per cent suspension in distilled water with 0.1 per cent sodium azide. The cells could be kept for at least one year without any apparent change in their properties. The formaldehyde treatment was a critical point in the PHI test. The blood from some donors gave better formaldehyde treated cells than did the blood from other donors. The reason for this is not known. Too short treatment resulted in unstable cells and too long treatment in sticky cells causing false positive agglutination patterns.

Tannic acid treatment was applied by incubating a 4 per cent suspension of formaldehyde treated red cells for 45 minutes at 56 C with an equal volume of tannic acid diluted 1/70 000. A 2 per cent suspension of cells washed 4 times was incubated for 120 min at 56 C in the immunoglobulin solutions which contained the following optimal concentrations 0.1 mg per ml for IgG or IgM and between 0.05 and 0.5 mg per ml for IgA. After washing 4 times with PBS the cells were stored at 4 C as a five per cent suspension in NRS diluted 1/100 with PBS.

Performance of the PHI test Two pre dilutions differing in concentration 1:1.26 were prepared from the sample and the standard. Starting from these tubes two 1.58 fold dilution series were made by means of a special syringe (for details see *Wide* (39, p. 28)); in this way one 1.26 fold ($10^{0.1}$) dilution series was obtained where every second tube originated from one of the two pre dilutions. Eight agglutinating doses of antibody and 0.5 per cent coated red cells were added to each tube. The set up for immunoglobulin determination consisted of the dilution series of the sample and the standard and controls. The positive control contained antiserum and coated cells; controls for unspecific agglutination contained coated cells suspended both in PBS and in the pre dilution of the sample but without antiserum.

The haemagglutination patterns were read after 60–90 minutes. The immunoglobulin concentration in the sample was calculated according to the formula given below. The $10^{0.1}$ construction of the dilution series facilitated the calculations, 10 tubes being equal to a 1:10 dilution.

$$C_x = \frac{C_s \cdot P_s}{P_x} \cdot 10^{-\frac{T_x - T_s}{10}}$$

where x stands for the sample and s for the standard, C for the immunoglobulin concentration, P for the first pre dilution and T for the tube number in the dilution series.

Standard serum To avoid denaturation of the protein used as a reference standard only fresh preparations were employed. Foaming and concentration were prevented. These standards were applied to determine by the SRD technique the immunoglobulin levels of a normal serum. Subsequently this serum served as a standard. Serum aliquots were kept at −20 C. Repeated thawing and freezing were avoided.

EXPERIMENTAL STUDY

Testing the PHI Method

In order to test the specificity of the antisera and immunoglobulin coated cells serial doubling dilutions of each antiserum were prepared and cells coated with IgG, IgA and IgM respectively were added. The results of such an experiment are shown in Table 1. They demonstrate that the cells are only agglutinated by the homologous antiserum. In gel filtration experiments the distribution of each immunoglobulin determined by the PHI test agreed well with results obtained by the SRD technique, the Ouchterlony tests and the immuno electrophoretic analyses. Thus there were no indications that proteins with gel filtra

tion behaviour other than that of the specific immunoglobulin interact with the test. Such experiments are shown in detail in a previous publication (21). Reasonable agreement between the values obtained by the PHI and paper electrophoresis methods were obtained when 10 different sera with M components of each of the immunoglobulin classes G, A and M were tested.

TABLE 1

Titres of Anti Immunoglobulin Sera Tested with Immunoglobulin Coated Cells

Cells coated with	Anti IgG	Anti IgM	Anti IgA
IgG globulin	80 000	< 20	< 20
IgM globulin	< 20	90 000	< 20
IgA globulin	< 20	< 20	2560

The sensitivity of the PHI method was calculated from the lowest concentration of the standard solution that caused complete inhibition. The mean value of ten consecutive analyses for each immunoglobulin was IgG 0.6 µg per ml, IgA 0.1 µg per ml and IgM 0.5 µg per ml respectively. By using 2–4 agglutinating doses of antiserum instead of 8 doses and taking 1.0 ml of the sample instead of 0.2 ml, it was possible to increase the sensitivity by about ten times. This, however, also increased the determination error. In analyses of undiluted serum and other solutions with high protein concentration, agglutination was sometimes less distinct.

The variation within one experiment was calculated from the difference between duplicate analyses of the three immunoglobulins in a normal population. The coefficient of variation (standard deviation in percentage of mean) was 19.5 per cent for IgG, 22.7 per cent for IgA and 19.3 per cent for IgM. The variation between different experiments was calculated from the values obtained for two control sera which were determined each time. The coefficient of variation was 23.4 per cent for analyses of IgG.

Testing of the SRD Method

The specificity of the SRD method was tested by means of different antigen preparation. In no case were measurable quantities obtained when an antiserum plate was tested with antigens of another class of immunoglobulins. The specificity was also verified in gel filtration experiments (17).

The antisera used gave the expected immunoglobulin concentration when purified IgG myeloma protein of the four different subgroups were tested. In a series of purified IgM and IgA M components there was a good agreement between values obtained by the SRD technique and those obtained by quantitative chemical determinations.

The sensitivity of the SRD method was regarded as the lowest concentration of the respective standard serum that produced a measurable

precipitin disk *i e* having a diameter 1 mm greater than that of the sample hole In the set up for routine work and expressed as the mean of ten analyses the lower limit of determination was for IgG 25 μg per ml for IgA 14 μg per ml for IgM 58 μg per ml and for IgD 27 μg per ml By application of 5 μl of the sample instead of 2 μl the lower limit of IgD determination was 10 μg per ml On application of undiluted serum to the agar plates a non specific precipitin disk was sometimes obtained which was possibly due to the presence of lipoproteins in the serum This phenomenon was less pronounced if the phosphate buffer was used (27) instead of the barbital buffer originally described by *Mancini et al* (23)

The precision of the SRD method was determined by dublicate analysis of 15 samples on the same plate from the same pre dilution The coefficient of variation was 5 1 per cent When the error was calculated from the values of the control serum which was tested each time the coefficients of variation were for IgG (29 plates) 9 7 per cent for IgA (16 plates) 7 2 per cent for IgM (17 plates) 7 4 per cent and for IgD (13 plates) 14 9 per cent The high figure for IgD was most probably due to double application of the sample in some of these tests and to the fact that many of the values were near the lower end of the standard curve

Standardization of the Immunoglobulin Level

The following preparations were used as standards two purified monomeric 7 S IgG normal protein preparations two purified monomeric 7 S IgA myeloma protein preparations two purified IgM M components and one purified IgD myeloma M component The comparisons

TABLE 2

Determination of the Immunoglobulin Levels in a Normal Serum Using Standards from Three Different Laboratories

	IgG		IgA		IgM	
	mg per 100 ml	Diff	mg per 100 ml	Diff	mg per 100 ml	Diff
Own standard	1190	–	125	–	141	–
Standard B	1190	0	213	+88	178	+37
Standard S	1250	+60	174	+49	135	–6

TABLE 3

Immunoglobulin Levels as Determined by the Passive Haemagglutination Inhibition Technique (PHI) and the Single Radial Diffusion Technique (SRD)

	IgG	IgA	IgM
PHI	1506 ± 446	164 ± 59 6	104 ± 50 6
SRD	1323 ± 279	158 ± 60 5	88 4 ± 43 4

TABLE 4

Immunoglobulin Levels in a Normal Swedish Population

	Number of samples	IgG Geometric Mean [± 2 SD]	IgG Arithmetic Mean (Range)	SD	IgA Geometric Mean [± 2 SD]	IgA Arithmetic Mean (Range)	SD
20–35 years							
men	12	1195 [729–1979]	1237 (803–1764)	291	112 [34.2–366]	138 (50.8–267)	65
women	14	1160 [765–1760]	1267 (756–1688)	277	123 [78.7–193]	126 (82.5–185)	29
36–50 years							
men	13	1330 [873–2030]	1387 (1056–2210)	319	165 [82.7–327]	181 (101–337)	113
women	14	1440 [877–2370]	1454 (961–2037)	340	167 [94.7–294]	170 (101–264)	47
51–70 years							
men	18	1230 [925–1630]	1240 (674–1588)	172	170 [80.2–360]	182 (99.4–379)	74
women	18	1330 [879–2000]	1355 (910–1961)	275	158 [60.3–413]	151 (89.1–212)	41
Men	43	1248 [834–1869]	1288 (874–2210)	237	148 [57.4–382]	167 (50.8–379)	78
Women	46	1315 [832–2079]	1359 (756–1961)	296	149 [69.9–318]	149 (82.5–264)	39
All	89	1281 [832–1973]	1323 (756–2210)	279	149 [63.2–350]	158 (50.8–379)	60

	Number of samples	IgM Geometric Mean [± 2 SD]	IgM Arithmetic Mean (Range)	SD	IgD§ Geometric Mean [± 2 SD]	IgD§ Arithmetic Mean (Range)	SD
20–35 years							
men	12	58.8 [26.4–131]	64.5 (30.8–107)	27.8	6.0 [1.4–25.8]	7.6 (2.1–17.9)	5
women	14	108 [51.7–226]	111 (54.3–159)	39.8	5.6 [2.4–13.5]	6.2 (3.0–15.0)	3
36–50 years							
men	13	67.6 [24.1–190]	73.4 (24.1–160)	37.4	4.6 [1.2–17.3]	5.9 (1.9–18.4)	5
women	14	94.0 [31.4–281]	108 (37.0–272)	60.[illegible]	6.6 [2.0–21.6]	7.8 (3.1–19.6)	5
51–70 years							
men	18	69.5 [18.2–265]	86.9 (20.6–279)	63.8	5.0 [1.3–18.3]	6.3 (2.2–23.8)	5
women	18	78.1 [30.5–200]	86.3 (31.1–160)	33.8	3.0 [1.7–5.3]	3.1 (1.7–4.5)	0
Men	43	65.4 [22.1–194]	74.9 (20.6–279)	49.7	5.1 [1.4–19.6]	5.6 (1.9–23.8)	5
Women	46	90.8 [34.9–236]	102 (31.1–169)	44.1	4.7 [1.6–14.1]	5.5 (1.7–10.6)	3
All	89	77.0 [26.4–224]	88.4 (20.6–279)	43.4	4.9 [1.5–16.7]	6.1 (1.7–23.8)	4

These limits are calculated via the log valu ... deter ... in ... ions.

§ IgD values less than 1 mg per 100 ml are r ...

between each of these pairs of standards fell within the error of the method. These standards were used to determine the immunoglobulin levels of a normal serum. Because of the risk of deterioration of the purified preparations during purification and storage, aliquots of the normal serum was used as a standard in the following. This standard serum was further tested against standard sera from two other laboratories. As is seen in Table 2, the IgG, IgM and one of the IgA values corresponded well, whereas the other IgA standard serum was much higher. The reason for this descrepancy is not clear.

RESULTS

Immunoglobulin levels in normal sera. Comparative investigations of serum from 43 men and 46 women between the ages of 20 and 70 were made by means of the PHI and SRD tests. The levels of IgG, IgA and IgM are presented in Table 3, which shows an acceptably good correlation between the two methods. Moreover, the IgD levels were determined by the SRD method.

Because of the rather large error of the PHI method, only the analytical results obtained by the SRD method are reported in detail. The results are shown in Table 4 and Fig 1. The IgG and IgA concentrations

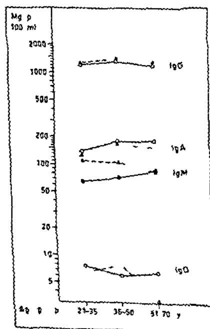


Fig 1

Mean levels of serum immunoglobulins G, A, M and D in men and women in different age groups. Circles and straight lines: men. Triangles and interspaced lines: women.

observed in the sex groups and the age groups did not differ whereas differences in IgM and IgD were observed In the age groups 20–35 and 36–50 the mean concentration of IgM was significantly higher in women than in men ($P<0.01$) The mean IgD for women was significantly lower in the age group 51–70 than in the age groups 20–35 ($P<0.001$) and 36–50 ($P<0.001$) The frequencies of cases of undetectable IgD were 3 men and no women out of 26 (12 per cent) in age group 20–35 3 men and 3 women out of 26 (23 per cent) in age group 36–50 and 3 men and 6 women out of 36 (25 per cent) in age group 51–70

Studies on subjects lacking IgA The frequency in the Swedish population of deficiency in IgA (less than 1 mg per 100 ml) was investigated using the PHI and SRD tests and the double diffusion in gel technique The methods were adjusted to detect concentrations above 1 mg per 100 ml In the sera from 3957 blood donors 9 (0 23 per cent) did not contain detectable IgA In the sera from 1401 pregnant women 3 (0 21 per cent) lacked IgA

The serum concentration of the other immunoglobulins in these subjects with IgA agammaglobulinaemia is shown in Table 5 The mean concentration of IgG was significantly higher ($P<0.001$) than in normals Eight of the 16 subjects deficient in IgA also lacked IgD which frequency (50 per cent) is higher than that in normal subjects (20 per cent) $\chi_1^2 = 4.954$ $p<0.05$ Compared with normal persons the IgA agammaglobulinaemic subjects did not show any significant differences in the IgM level

TABLE 5

Immunoglobulin Concentrations in Sera from Individuals Lacking IgA

	Age years	Classification	Sex	Levels in mg/100 ml IgD	IgG	IgM
I S	21	Healthy	♀	7 3	1470	118
B.J	35	Healthy	♂	3 4	2550	98
E B	41	Healthy	♂	4 3	1170	73
B R	45	Healthy	♂	< 1	2490	106
L.B	20	Healthy	♂	3 4	1810	79
L.J	20	Healthy	♂	< 1	3020	112
B K	25	Healthy	♂	22	1220	44
F K	25	Healthy	♂	1 7	1710	39
J R	32	Healthy	♂	< 1	1200	67
L.H	29	Healthy	♂	2 1	1770	86
M Su	31	Pregnant	♀	< 1	1660	86
M Sa	26	Pregnant	♀	1 7	1580	77
S S	21	Pregnant	♀	< 1	1620	64
C S	38	High ESR	♂	< 1	1290	118
K A †	63	Ulcus varicosum	♂	< 1	1900	97
V L †	62	Asthma	♂	< 1	1800	81
		Arithmetic mean ± 1 S D		5 7 ± 1 9§	1767 ± 592	84 ± 21

Detected in other laboratories Not included in the frequency study

† Detected in routine investigation on patients Not included in the frequency study

§ IgD values less than 1 mg per 100 ml are not included in the calculations

Studies on subjects lacking IgD In the healthy persons the frequency of IgD agammaglobulinaemia (less than 1 mg per 100 ml) was 12 per cent 23 per cent and 25 per cent in three age groups As previously mentioned a higher frequency 30 per cent was observed in subjects lacking IgA In order to investigate a possible genetic influence the IgD level was determined in 56 pairs of monozygotic twins and in 51 pairs of dizygotic twins The frequency of IgD deficiency was 16 per cent in the monozygotic twins and 19 per cent in the dizygotic The difference between the IgD levels of each pair of twins was plotted In the mono zygotic twin group 72 per cent presented a difference of 2 mg per 100 ml or less whereas the corresponding figure for the dizygotic twin group was 57 per cent A more detailed study of IgD and of immuno globulins in twins will be reported separately

DISCUSSION

With good antisera sufficiently absorbed and with appropriate controls both of the methods tested gave specific results The PHI technique was about 50–150 times more sensitive than the SRD method and the results more rapidly obtained but the determination error was about 2–3 times greater Consequently the SRD technique was considered more suitable for quantitative determinations of immunoglobulins G A M and D in the concentration range usually of interest for clinical diagnosis

Compared with the method originally described (23) some simplifi cations of the SRD technique have been introduced Although this re sulted in a determination error greater than that reported by *Mancini et al* the technique was more suitable for clinical routine The PHI technique is of great value when high sensitivity is required It has been used for the determination of different immunoglobulins on anti body coated cells and in eluates containing antibodies (15) The fact that non specific reactions were sometimes obtained when low dilu tions of serum were tested made the method less useful for the deter mination of low concentration serum proteins than could have been expected from the high sensitivity of the method as such Recent studies indicate that an even more sensitive radio immunologic technique may be more suitable for this purpose

The present report deals with normal immunoglobulin levels in adults The concentrations in children up to five years of age are given elsewhere (18) Due to the fact that the biological variation does not show a typical gaussian distribution especially as far as IgM and IgD are concerned the mean and the standard deviation were also calculated from the logarithmic values The geometrical mean and the antilog for the two standard deviation limits are presented in Table 4 and seem to give the most realistic fit for clinical use The levels of IgG IgM and IgD tally well with those reported by other authors (3 4 6 7–14 20 22

24 25 27, 28 30 31 33) A comparison between results obtained in some laboratories has been published by *Kohler & Farr* (22) *Butterworth et al* (6) have recently demonstrated that the IgM level is the same in boys and girls before puberty but is subsequently higher in females In the present investigation the IgM level was found to be higher in women of fertile age but after the menopause it was the same as in men (see Fig 1) These findings may indicate a sex hormonal influence on the regulation of immunoglobulin M

The normal concentration of IgA varies more in different reports This may be due to the well known difficulties in preparing a good reference standard (25) The normal level reported in the present paper agrees with the levels stated in (8 25 28) It is well known that healthy persons may lack detectable amounts of IgA (26) However only few reports concerning the frequency of this defect have apparently been published (2) In the present study the frequency at which lack of IgA was encountered (in 0 23 per cent among blood donors and in 0 21 per cent among pregnant women total 0 22 per cent) is somewhat higher than the frequency of 0 14 per cent found by *Bachman* (2) The increased concentration of IgG in the cases of IgA agammaglobulinaemia may be the result of a compensatory mechanism No cause is known to account for the increased frequency at which lack of detectable IgD concentration occurs in persons with IgA agammaglobulinaemia It may however indicate the presence of a common control mechanism of the immunoglobulin synthesis It seems as if the distribution of the IgD levels indicates the existence of *two populations* one with concentrations below 1 mg per 100 ml and one with a geometric mean of 4 9 and a lower confidence limit (mean minus 2 S D) of 1 5 mg per 100 ml This indicates a possible genetic influence Further support for this interpretation was obtained in a preliminary study of twins

SUMMARY

Quantitative determination of human immunoglobulins has been made with the aid of two methods a passive haemagglutination inhibition technique (PHI) and a single radial diffusion technique (SRD) The PHI test allowed estimation of immunoglobulin concentrations as low as 0 1 μg per ml the corresponding figure for the SRD test was 14 μg per ml The coefficient of variation of the PHI test was about 20 per cent and that of the SRD test about 5 per cent In clinical laboratory routine the SRD technique is more suitable for a determination of the immunoglobulins G A M and D and in other instances when extremely high sensitivity is not required For the latter purpose the PHI test is useful

In a group of 89 healthy persons selected as representing a normal Swedish population the following immunoglobulin levels were obtained The values are presented as arithmetic and geometric means in

mg per 100 ml with confidence limits expressed as ± 2 S D Arithmic values IgG 1323 (765–1881) IgA 158 (37–279) IgM 88 4 (1 6–175) and IgD 6 1 (0–15 5) Geometric values IgG 1281 (832–1973) IgA 149 (63 2–350) IgM 77 0 (26 4–224) and IgD 4 9 (1 5–16 7)

The mean IgM concentration was significantly higher in women than in men in the age groups 20–50 In women the mean IgD concentration was significantly lower in the age group 51–70 compared with the age groups 20–35 and 36–50 The number of women with non-detectable IgD increased with increasing age

The frequency of non detectable IgA was 9/3957 (0 23 per cent) among blood donors and 3/1401 (0 21 per cent) among pregnant women (total 0 22 per cent) The concentration of IgG was significantly higher in the subjects with IgA agammaglobulinaemia than in normal persons Eight out of 16 individuals lacking IgA were deficient also in detectable IgD a frequency which was higher than that in normals (20 per cent) The possibility of a common control mechanism in the synthesis of IgA and IgD was postulated This was supported to some extent by a study of IgD in monozygotic and dizygotic twins

REFERENCES

1 *Axelsson U Bachmann R & Hallen J* Frequency of pathological proteins (M-components) in 6 995 sera from an adult population Acta med scand 179 235–247 1966

2 *Bachmann R* Studies on the serum γA-globulin level III The frequency of A γA globulinemia Scand J clin Lab Invest 17 316–320 1965

3 *Bachmann R* The serum γM globulin level in healthy subjects hypergamma globulinemic patients and conditions with pathological proteins (M components) in serum Scand J clin Lab Invest 18 280–288 1966

4 *Bachmann R & Laurell C B* Studies on the serum γ_1A globulin level I Health ... ojects and hypergammaglobulinemic patients Scand J clin Lab Invest 17 39 45 1965

Boyden S V The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera J exp Med 93 107–120 1951

6 *Butterworth M McClellan B & Allansmith M* Influence of sex on immunoglobulin levels Nature 214 1224–1225 1967

7 *Chodirker W B & Tomasi Jr T B* Gamma globulins Quantitative relationships in human serum and nonvascular fluids Science 142 1080–1081 1963

8 *Claman H N & Merrill D* Hypergammaglobulinemia—the role of the immune globulins (γG γA γM) J Allergy 36 463–471 1965

9 *Collins Williams C Tkachyk S J Toft B & Moscarello M* Quantitative immunoglobulin levels in children Int Arch Allergy 31 94 103 1967

10 *Fahey J L & Lawrence M E* Quantitative determination of serum immuno β_2A globulins and γ_1 macroglobulins in human serum J Immunol 91 ... 1963

11 *Fahey J L & McKelvey E M* Quantitative determination of serum immunoglobulins in antibody agar plates J Immunol 94 84–90 1965

12 *Haferkamp O Schlettwein Gsell D Schwick H G & Störiko K* Serum protein in aging population with particular reference to evaluation of immune globulins and antibodies Gerontologia 12 30–36 1966

13 *Heremans J F* Les globulines serique du systeme gamma Ed Arscia Brussels 1960

14 *Hundley C C & Lyerly A* Immune globulin determinations in allergic children Amer J Dis Child 106 545–552 1963

15 *Hogman C F Killander J & Johansson S G O* Identification of immunoglobulins with antibody activity fractionated by gel filtration Vox Sang 9 10–13 1964
16 *Johansson S G O & Bennich H* Immunological studies of an atypical (myeloma) immunoglobulin Immunology (Lond) 13 381–394 1967
17 *Johansson S G O Bennich H & Wide L* A new class of immunoglobulins in human serum Immunology 14 265–272 1968
18 *Johansson S G O & Berg T* Immunoglobulin levels in healthy children Acta paediat scand 56 572–579 1967
19 *Johansson S G O Hogman C F Killander J & Wide L* Quantitative determination of γ_1M γ_1A and 7S γ globulins by a modified Boyden technique Acta path microbiol scandinav 60 303–304 1964
20 *Keimowitz R J* Immunoglobulins in normal human tracheobronchial washings A qualitative and quantitative study J Lab clin Med 63 54–59 1964
21 *Killander J* Separation of human immunoglobulins by gel filtration and zone electrophoresis Acta Soc Med Upsal 68 230–244 1963
22 *Kohler P F & Farr R S* Quantitative comparison of immunoglobulins in atopic (reaginic) and nonatopic (nonreaginic) individuals Higher γD levels in atopic sera J Allergy 39 311–322 1967
23 *Mancini G Carbonara A O & Heremans J F* Immunochemical quantitation of antigens by single radial immunodiffusion Immunochemistry 2 235–246 1965
24 *McKelvey E M & Fahey J L* Immunoglobulin changes in disease Quantitation on the basis of heavy polypeptide chains IgG (γG) IgA (γA) and IgM (γM) and of light polypeptide chains type K (I) and type L (II) J clin Invest 44 1778–1787 1965
25 *Norberg Renée* The immunoglobulin content of normal serum Acta med scand 181 485–496 1967
26 *Rockey J H Hansson L A Heremans J F & Kunkel H G* Beta 2A aglobulinemia in two healthy men J Lab clin Med 63 205 212 1964
27 *Rowe D S & Fahey J L* A new class of human immunoglobulins II Normal serum IgD J exp Med 121 185 199 1965
28 *Stiehm E R & Fudenberg H H* Serum levels of immunoglobulins in health and disease a survey Pediatrics 37 715–727 1966
29 *Weinbach R* Die Verwendbarkeit formolbehandelten Erythrocyten als Antigentrager in der indirekten Haemagglutination Schweiz Z Path Bakt 21 1043 1052 1958
30 *Weiss W A* Normalwerte spezifischer Serumproteins im Kindes und Erwachsenenalter Klin Wschr 43 273–277 1965
31 *West C D Hong R & Holland N H* Immunoglobulin levels from newborn period to adulthood and in immunoglobulin deficiency states J clin Invest 41 2054 2064 1962
32 *Wide L* An immunological method for the assay of human chorionic gonadotrophin Acta endocrin Kbh 41 Suppl 70 1962
33 *Wollheim F A & Williams Jr R C* Studies on the macroglobulins of human serum I Polyclonal immunoglobulin class M (IgM) increase in infectious mononucleosis New Engl J Med 274 61 67 1966

Acta path. microbiol. scandinav. 74: 531–542, 1968

The Department of Medical Microbiology (Head: Professor R. Grubb), University of Lund, Lund, Sweden

PRODUCTION OF ANTIBIOTICS BY *EPIDERMOPHYTON FLOCCOSUM*

2. *Microflora in Epidermophyton Infected Skin and its Resistance to Antibiotics Produced by the Fungus*

By

ALF WALLERSTRÖM

Received 24.iv.68

Opinions differ as to whether antibiotics occur in nature in ecologically significant amounts. The lack of unanimity is probably due mainly to the difficulty in demonstrating these substances in low concentrations in the natural microenvironments of the microbes and consequent discrepancies between the results obtained by different investigators. The production of *inter alia* gliotoxin, chloramphenicol, actinomycin and griseofulvin has been demonstrated in sterilized soil inoculated with antibiotic producing strains (*Brian* 1957), but in soil containing not only the antibiotic producer but also normal microflora such experiments have as a rule been unsuccessful. *Wright* (1954, 1956) however demonstrated that gliotoxin was formed locally in buried fragments of plant debris and also demonstrated the substance in unsterilized soil enriched with other organic material such as clovermeal. Antibiotics from plant parasites (patulin, alternaric acid, fusaric acid) have been found in affected plant tissue (*Brian* 1957). It has been postulated that parasitic micro-organisms produce antibiotics in human tissue, e.g. by *Aspergillus fumigatus* in pulmonary aspergilloma (*Segretain* 1962), but supportive evidence appears to be lacking. The formation of antibiotics *in vitro* does not necessarily mean that such production occurs also *in vivo*. *In vitro Pseudomonas aeruginosa* forms pyocyanin which is active against *inter alia* staphylococci, but in mixed infections the two microbes are claimed to act synergistically (*Caminiti* 1939).

Patients with dermatomycosis are fairly often allergic to penicillin (*Lorma & Lewis* 1946, *Götz & Thies* 1952, *Blum & DeWeck* 1966). *Peck & Hewitt* (1945) showed that dermatophytes, especially *Trichophyton mentagrophytes*, form a penicillin-like substance *in vitro*. In a later publication, *Peck* (1950) stated that this explains the mechanism by which dermatophytons may induce a positive penicillin test. *Orr et al.*

(1955) and *Cole* (1966) showed that strains of *Trichophyton mentagrophytes* as well as *Epidermophyton floccosum* in shake culture were able to produce several kinds of penicillin *inter alia* penicillin G F and dihydro F In a later paper *Ort et al* (1957) reported experiments in which pieces of epithelium infected with dermatophytes gave rise to zones of inhibition in plates inoculated with *B subtilis* When penicillinase was added to the substrate the zones were smaller or did not occur These results were obtained in experiments with *Trichophyton mentagrophytes* and *Epidermophyton floccosum* in experiments with *Trichophyton violaceum* no zones of inhibiton appeared

In a previous investigation (*Wallerstrom* 1967) it was shown that culture filtrate of *Epidermophyton floccosum* contains not only penicillin but also an unidentified antibiotic which is distinguishable from penicillin on its antibiotic spectrum Since resistance to the latter antibiotic (below called EPF = Epidermophyton factor) proved to be rare among staphylococci from clinical material it was decided to study the susceptibility of those staphylococci and micrococci that could be cultured from mycotic foci due to *Epidermophyton*

MATERIAL AND METHODS

Material

The material consisted of skin scrapings from 37 patients where previous or simultaneous culture had given growth of *Epidermophyton floccosum* The clinical diagnosis was tinea inguinalis in 27 of the case tinea pedis in 7 tinea corporis in 2 and tinea manu in 1

As controls were used corresponding specimens from 35 persons which were examined on clinical suspicion of mycosis but in whom culture for fungi had given no growth of dermatophytes Twenty of these (a series studied with a view to estimating the frequency of tinae pedis in a group of military recruits) were re examined 12 days later with a new culture and 10 of them a third time within some further week All these cultures proved negative for dermatophytes The samples from these recruits had been obtained from the toe webs In the remaining controls the material had been obtained from the groin in 3 from glabrous skin in 2 from the foot in 5 and from the hand in 5 The mean age of the patients in the *Epidermophyton* group was 25 8 years that of the controls 26 2 years

Methods

Cultivation and examination of bacteria 24 specimens of skin scrapings from lesions due to *Epidermophyton floccosum* were cultured for bacteria immediately after their arrival at the laboratory The remaining 13 specimens were cultured for bacteria after culture for fungi had proved positive The interval between receipt of the specimen and the culture for bacteria never exceeded 14 days As to the control material 23 specimens were cultured for bacteria immediately and 12 not until culture for fungi had proved negative *i e* after 14 days

The samples were inoculated into both solid and liquid media All growth was analysed according to conventional bacteriological principles *Corynebacterium* spp were, however excluded from the investigation (see under Discussion) Colonies of staphylococci and micrococci to be tested for susceptibility to antibiotics were taken as far as possible from growth on solid medium and only if the number of colonies was small was the *growth in liquid medium* used Depending on the number of staphylococcus like colonies 3–10 colonies from each sample were examined (median value in the *Epidermophyton* material 8 4 and in the control material 8 3) —The *resistant strains* tendency to spread to other areas of the skin was studied in a few cases by culturing material from the actual mycotic foci as well as from un

affected areas (nose axilla sternal region a o) and the foci were repeatedly ex amined for growth of fungi and bacteria during treatment of the patients

Staphylococcus like strains were examined for coagulase activity in the tube coa ulati n tests and for their capacity to ferment glucose by culture on special medium (see below) in anaerobic jar at 37 C for 2 days

D termination of the susceptibility to EPF and penicillin All is lates of bacteria were tested for susceptibility to EPF by a plate diffusion technique cylindrical holes 8 mm in diameter were punched out in plates inoculated with the bacteria to be tested and the holes were then filled with culture filtrates of a standard strain of *Epidermophyton* (test strain No 1 Wallerström 1967) The inhibition zones were measured after 17-20 hours incubation of the plate at 37 C. With a reference strain of *Staphyl coccus albus* the standard filtrates produced inhibition zones of 30 mm in diameter

The susceptibility of the bacteria to penicillin was studied by means of an agar diffusion test performed with paper disks containing 20 IU benzyl penicillin (sup plied by Bacteriologiska Laboratoriet Karolinska Sjukhuset Stockholm) (*Ericsson* 1960) Only part of the material was tested for susceptibility to penicillin (strains from 29 individuals in the *Epidermophyton* material and 23 of the controls)

Microbes giving zones more than 15 mm in diameter were classified as sensitive to EPF (see Fig 1 and text under Results) Susceptibility to penicillin was classified as follows zone 5-11 mm = resistant, zone 12-20 mm = slightly sensitive zone 21-30 mm = m derately sensitive zone 31 mm or more = sensitive The term decreased sensitivity is to be understood a resistant or slightly sensitive

Production of Epidermophyton filtrates From each of the 37 *Epidermophyton* strains isolated from the samples one or several shake cultures in broth were pre pared with a technique described earlier (*Wallerstrom* 1977) The filtrates of the standard strain were produced in the same way The broth was filtered through a Seitz EK and the filtrates were studied for their effect on the reference staphylo coccus In 14 cases bacteria from *Epidermophyton* lesions were examined both with filtrate of the standard strain and with filtrate of the fungus found in the same clinical specimen

Media for culture of fungi Each specimen was inoculated on one agar plate con taining 4 per cent horse blood and on two plates with Sabouraud medium (Oxoid) one f which contained 0 1 per cent cycloheximide All of these media contained 0 IU penicillin and 50 µg of streptomycin per ml

Media for bacterial culture The solid medium c nsisted of agar plates containing Blood Agar Base No 2 (Oxoid) and 4 per cent horse blood the liquid medium of trypton broth containing 0 1 per cent thioglycollate DST agar (Oxoid) was used in tests for susceptibility to antibiotics In the estimation of the capacity of bacteria to ferment glu ose (used for the purpose of distinguishing staphylococci from micro cocci) u e was made of a medium of the following composition

	an trade pepton water (Oxoid)	15 g
	glucose	10 g
	agar	10 g
	distilled water	1000 g
pH 9		

Clinical data of Epidermophyton infected individuals The hospital records and other information from the patients doctors were studied For each patient was noted the site of the mycotic lesion as well as its extent and duration the presence of mycids (microbids) local treatment given (antibacterial antimyc tic cortison derivatives) and the patient s ag and occupation.

RESULTS

Definition of the Term 'Resistant' to EPF

In an earlier publication (*Wallerstrom* 1967) bacterial strains with an inhibition zone more than 15 mm in diameter were arbitrarily de

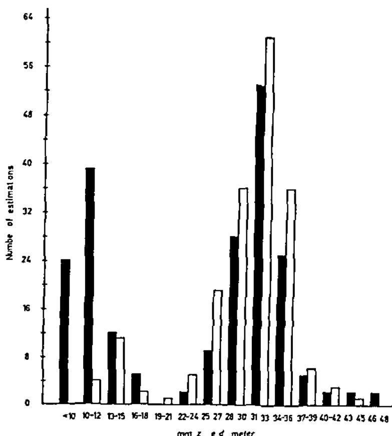


Fig 1

Sensitivity to EPF of staphylococci and micrococci from *Epidermophyton* lesions and a control material Distribution of zone diameters in agar plate diffusion test
Filled columns strains from the *Epidermophyton* material
Blank columns strains from the control material

signated sensitive to EPF The mean value of zone diameters for 30 staphylococcal strains in this study was 27 4 mm (range 24–32 mm)

184 colonies of staphylococci and micrococci from the control material and 206 colonies from the *Epidermophyton* material were isolated and their susceptibility to EPF was determined by the plate diffusion method The resulting zone diameters are recorded graphically in Fig 1

It is clear from the figure that the distribution was bimodal a zone diameter of 15 mm being well below the mean value for the staphylococci and micrococci tested

Frequency of Microbes with Decreased Sensitivity for EPF and Penicillin in Mycotic Foci Caused by Epidermophyton

Among the 37 specimens from mycotic foci bacterial growth was obtained in 36 In one case growth was obtained of *E coli* only and in one case of enterococci only The remaining 34 cultures gave growth

of staphylococci and/or micrococci Twelve cases in addition gave growth of various yeasts gram negative rods enterococci and non haemolytic streptococci

TABLE 5

Microbial Flora in Skin Specimens from Epidermophyton Infected Individuals Classification after Susceptibility to EPF and Penicillin

Staphylococci and micrococci	*No of patients*	Other microbes
Resistant to EPF sensitivity to penicillin decreased	*13*	gram negative rods (*) gram negative rods and streptococci (1) gram negative rods streptococci and yeasts (1)
Resistant to EPF moderately sensitive or sensitive to penicillin	*5*	gram negative rods (1) yeasts (1)
Sensitive to EPF sensitivity to penicillin decreased	*4*	enterococci and yeasts (1) yeasts (1)
Sensitive to EPF moderately sensitive or sensitive to penicillin	*7*	gram negative rods and yeasts (2) yeasts (2)
Resistant to EPF susceptibility to penicillin not investigated	*1*	
Sensitive to EPF susceptibility to penicillin not investigated	*4*	
No staphylococci or micrococci found	*3*	enterococci (1) gram negative rods (1)

Figures in brackets represent number of cases

EPF resistant staphylococci and/or micrococci were found in 19 out of 34 (55 9 per cent) of the specimens from patients with *Epidermophyton* infections The susceptibility to penicillin was studied in 29 cases in 17 (58 6 per cent) strains of these bacteria were found that showed decreased sensitivity to penicillin Strains with decreased sensitivity to both EPF and penicillin were found in 13 among 29 patients (44 8 per cent)

Coagulase positive staphylococci were found in 5 patients coagulase negative staphylococci in 27 and micrococci in 10 Twelve cultures contained more than one of these species of bacteria and 15 more than one strain of one of the species the strains differing from one another in susceptibility to EPF and/or penicillin

The frequency of strains with decreased sensitivity to penicillin was roughly the same among the coagulase positive staphylococci as among the coagulase negative and micrococci None of the 5 coagulase positive strains was resistant to EPF

In 8 cases culture for fungi gave growth not only of *Epidermophyton* but also of yeast like fungi (*Candida albicans C parapsilosis C guil*

lermondi and others) FPF resistant strains of staphylococci and micrococci were demonstrated in 2 of these cultures and in 3 of them strains of these microbes that were only slightly sensitive to penicillin

In 8 cases culture gave growth not only of staphylococci and micrococci but also of gram negative rods (*E coli*, *Enterobacter* sp, *Achromobacter* sp *Pseudomonas aeruginosa*) enterococci and non haemolytic streptococci As expected from the antibacterial spectrum of FPF (*Wallerstrom* 1967) these bacteria were resistant to this antibiotic The frequency of FPF resistant staphylococci and micrococci in cultures with simultaneous growth of other FPF resistant bacteria was not significantly higher than in the rest of the *Epidermophyton* material (Table 1)

Among the 22 cases which harboured staphylococci or micrococci with decreased sensitivity to FPF and/or penicillin strains which were sensitive to both antibiotics were found in addition in 14 In 8 cases were strains with decreased sensitivity to FPF and penicillin found together with strains with decreased sensitivity to only one of these antibiotics

Filtrates from 14 *Epidermophyton* strains were tested against the bacteria isolated from the same clinical specimens as the fungi in question In no instance did the results differ from those obtained with filtrate from the standard strain

Frequency of Microbes with Decreased Sensitivity to FPF and/or Penicillin in the Control Material

Among the 35 cultures from the control material growth was obtained in 32 One sample contained only coagulase positive staphylococci all the others contained coagulase negative staphylococci and 10 of these samples gave growth of micrococci too In 17 cases various yeasts gram negative rods and enterococci were found in addition

FPF resistant staphylococci and/or micrococci were found in 4 among 32 individuals (12 5 per cent) The susceptibility to penicillin was studied in 22 cases and in 4 (18 2 per cent) strains of these bacteria were found that showed decreased sensitivity to penicillin All of the strain with decreased sensitivity to both FPF and penicillin All of the FPF resistant strains and 3 of the 4 strains with decreased sensitivity to penicillin were found in those samples that derived from military recruits

Culture of control material for fungi gave no growth of dermatophytes but in 16 cases growth (usually scanty) was obtained of yeast like fungi (*Candida parapsilosis* *C guillermondi* *Torulopsis* and *Trichosporon* spp) All of the 4 FPF resistant strains and 2 of the 4 strains with decreased sensitivity to penicillin derived from samples that had also given growth of yeastlike fungi

TABLE 2

Microbial Flora in Skin Specimens from the Control Material Classification after Susceptibility to EPF and Penicillin

Staphylococci and micrococci	*No of patients*	*Other microbes*
Resistant to EPF moderately sensitive or sensitive to penicillin	4	enterococci and yeasts (1) yeasts (3)
Sensitive to EPF sensitivity to penicillin decreased	4	yeasts (2)
Sensitive to EPF moderately sensitive or sensitive to penicillin	15	enterococci (1) enterococci and yeasts (1) gram negative rods and yeasts (2) yeasts (3)
Sensitive to EPF susceptibility to penicillin not investigated	9	yeasts (2)
No staphylococci or micrococci found	3	

Figures in brackets represent number of cases

EPF resistant bacteria other than staphylococci or micrococci (*E coli Achromobacter* enterococci) were found in samples from 5 cases 4 of which also gave growth of yeasts (Table 2) EPF resistant staphylococci were found in one of these samples

Sensitivity of Microflora to EPF and Penicillin in Relation to Clinical Data

Staphylococci and micrococci with decreased sensitivity to EPF and/or penicillin were equally common in tinea inguinalis as in tinea pedis As to other forms of tina the cases were too few to warrant any conclusions regarding the frequency of resistant strains The frequency was found to be the same in mycotic foci that were small (described as being the size of a coin) as in those that were as large as the palm of the hand or larger

The occurrence of EPF resistant staphylococci/micrococci was found to vary inversely with the interval after the onset of the symptoms of the mycosis The material fell into two groups one where the interval was only 1–6 weeks and one where it covered several months or even a year or more (Table 3)

Among the cases in which the mycosis had lasted for 6 weeks or less the frequency of EPF resistant staphylococci and micrococci was significantly higher (75 per cent) than among the long standing cases where such strains were found in only 1 case out of 9 The frequency of strains with decreased sensitivity to penicillin showed the same tendency but the difference was not significant (Table 4)

TABLE 3

Occurrence of EPF Resistant Staphylococci and Micrococci in Epidermophyton Infected Skin in Relation to the Interval Since the Debut of Symptoms

Duration	Total number of patients	EPF res. strains	No EPF res strains
6 weeks or less	20	15	5
3 months ore more	9	1	8
(Interval unknown	5	3	2)
		$P < 0.01$	

TABLE 4

Occurrence of Staphylococci and Micrococci with Slight Sensitivity to Penicillin in Epidermophyton Infected Skin in Relation to the Interval Since the Debut of Symptoms

Duration	Total number of patients	Strains with decreased sensitivity found	Strains with decreased sensitivity not found
6 weeks or less	17	10	7
3 months or more	7	3	4
(Interval unknown	5	4	1)
		$0.95 < P < 0.1$	

The patients in whom the condition had lasted for less than 6 weeks were younger (mean 25.0 years) than those in whom the condition had lasted longer (mean 33.1 years). The material would not warrant any conclusions regarding the effect of any of sex and occupation.

On culture *in vitro* with the technique described previously (*Wallerstrom* 1967) the *Epidermophyton* strains were found to vary widely in their capacity to produce EPF. No correlation between this capacity and the appearance of EPF resistant microbes in the lesions produced by the fungus (Table 5) was found.

TABLE 5

Comparison between Epidermophyton Strains Activity in vitro against Staph albus (Reference strain) and the Occurrence of EPF Resistant Staphylococci/Micrococci in the Mycotic Lesions Caused by the Same Fungal Strains

Antibacterial activity of filtrate	EPF resistant strains Number of cases	No EPF resistant strains Number of cases
High (zone diameter ≧ 30 mm)	2	4
Moderate (zone diameter 21–29 mm)	11	6
Low (zone diameter ≦ 20 mm)	6	5

The bacterial flora outside the mycotic foci was examined in 3 cases Samples for culture were taken from the axilla or from the nose and sternal region and in one of the cases a patient with unilateral mycosis of the foot from the contralateral foot Staphylococci or micrococci were demonstrated in most of the samples but no resistant strain of the type found in the fungal foci were demonstrable except for a single finding in one out of three samples of the resistant strain in the patient s healthy foot

DISCUSSION

FPF resistant staphylococci or micrococci were found about 4 times as often in samples from *Epidermophyton* infected skin as in samples from control material and strains with *decreased sensitivity to penicillin* about 3 times as often as in the controls the difference is significant in both cases ($P<0\ 01$) Strains with decreased sensitivity to both EPF and penicillin were found in 13 of the *Epidermophyton* infected individuals but not at all among the controls which also makes a significant difference ($P<0\ 001$) The results suggest that FPF is produced by *Epidermophyton* in its natural environment and that this is valid also for the penicillin like substance

The frequency of microbes with decreased sensitivity to EPF and/or penicillin did not vary with the site of the mycotic focus or its absolute extent On the other hand the frequency of FPF resistant staphylococci/micrococci was significantly lower in cases of long duration of the mycosis than in cases which had lasted only for a few days or weeks No satisfactory explanation can as yet be offered for this phenomenon The average age of the patients in whom the condition had persisted for less than 6 weeks was significantly lower than that of the remainders Antibacterial treatment with reduction of the bacterial population may have played a role and at least 4 out of the 9 patients who had had the disease for a long time had received such treatment (the corresponding figures for those who had had the disease for a short time were 5 and 20) On the other hand follow up showed that resistant strains could persist for up to 1 month in spite of combined antibacterial antimycotic treatment.—The disappearance of resistant strains might be explained by the assumption that EPF is antigenically active and that in skin infected with fungi the substance is inactivated by an antigen antibody reaction An investigation of this possibility is planned

The poor correlation between the occurrence of EPF resistant staphylococci/micrococci and other FPF resistant microbes such as gram negative rods may be due to the smallness of the material but may also be connected with the fact that the latter bacteria are transients which occur in the skin in smaller numbers than the resident" staphylococci (*Kligman* 1965)

No correlation was found between the occurrence of staphylococci

or micrococci with decreased sensitivity to EPF or penicillin and the occurence of fungi other than *Epidermophyton* which rules out any effect of hypothetic antibiotic producers among the latter

The control material did not consist of symptomless individuals but of persons from whom samples had given no growth of dermatophytes In a few cases growth of *Candida parapsilosis* or *Candida guilliermondi* was fairly abundant and suggested the presence of a yeast dermatitis Neither in the mycotic group nor in the controls were the skin specimens examined microscopically In a compilation published by *Gotz* (1962) the results of culture and microscopic examination were concordant in on the average 80 per cent while in on the average 18 per cent fungi were found only at microscopy In most control cases in the present investigation samples for culture were collected on more than one occasion and it is unlikely that a case of dermatomycosis would have passed unnoticed

The frequency of EPF resistant staphylococci/micrococci in the control material was higher than expected In an earlier study on staphylococci originating from nose and throat swabs (*Wallerstrom* 1967) the incidence of EPF resistant strains was only 3 out of 466 *i e* 0 6 per cent these 3 strains were sensitive to penicillin The higher frequency of EPF resistant strains among the controls in the investigation may have an epidemiologic background as all the resistant strains were found in specimens from military recruits of one and the same regiment i e direct spread of resistant strains may have occurred It is also possible that the individuals with resistant strains on the feet had come into contact with dermatophytes on some earlier occasion the finding of dermatophytes on clinically normal feet has been reported by many authors most figures given ranging from 1 5 to 6 per cent (*Gotz* 1962)

The group of diphtheroids (*Corynebacterium* spp) were intentionally not included in the analysis of the bacterial flora in the mycotic foci though micro organisms of this genus are sensitive to EPF and though it might be of interest to analyse the possible occurrence of resistant variants This would however require a culture technique different from that used here because for example anaerobic strains have been described by some authors (*Evans et al* 1950) as constituting a considerable part of the skin flora and the frequency of lipophilic strains requiring a special medium is unknown (*Marples* 1965)

The results of the investigation point in the same direction as those in a study by *Smith & Marples* (1964) in which they showed that in dermatophyte infected animals (hedgehogs infected with *Trichophyton mentagrophytes var erinacei*) the staphylococcal strains that could be isolated from the skin were in 92 per cent resistant to penicillin the formation of penicillin was also demonstrated *in vitro* from the isolated fungus strains They concluded and their conclusion was shared by *Brock* (1966) that penicillin is formed by the fungus in these environ

ments and in such quantities that it can have a selective effect on the microflora of the skin

SUMMARY

The bacterial flora of foci of *Epidermophyton floccosum* from 37 patients with mycosis was studied with a view to the composition and sensitivity to penicillin and to an antibiotic which is produced by *Epidermophyton* and called EPF (*Epidermophyton* factor). Among the isolated staphylococci and micrococci 55 9 per cent were resistant to EPF and 58 6 per cent were only slightly sensitive to penicillin. The corresponding figures for the controls were 12 5 per cent and 18 2 per cent respectively. The frequency of EPF resistant strains was highest when the disease had lasted for no more than one month. The findings suggest that *Epidermophyton floccosum* produces antibiotics also *in vivo* and that the antibiotics produced have a selective effect on the skin flora

REFERENCES

1 *Brian P W* The ecological significance of antibiotic production In Microbial ecology 7th Symposium of the Society for general microbiology Cambridge University Press 1957 pp 168–188
2 *Blum G & De Weck A L* Über den Zusammenhang von Dermatomykosen mit der Penicillin Überempfindlichkeit Dermatologica *133* 461–472 1966
3 *Brock T* Principles of microbial ecology Prentice Hall Inc /Englewood Cliffs New Jersey 1966 pp *130–131*
4 *Caminetti S* quoted in *Elek S D* *Staphylococcus pyogenes* and its relation to disease F & S Livingstone Ltd Edinburgh & London 1959 pp 175–176
5 *Cole M* Formation of 6 aminopenicillanic acid penicillins and penicillin acylase by vari us fungi Appl Microbiol *14* 98–104 1966
6 *Cormia F F & Lewis G M* Experimental aspects of penicillin ensitization with special reference to conjoint sensibility to superficial fungus disease J Invest Dermat 7 375–380 1946
7 *Fries on H* Rational use of antibiotics in hospital Scand J Clin Lab Invest *1?* Suppl 50 1960
8 *Evans C A* *Smith W B* *Johnston F A* & *Giblett F R* Bacterial flora of the normal human skin J Invest Dermat *15* 305–324 1950
9 *Got H & Thies W* Zur Beurteilung des Ausfalls der intracutane Penicillinreaktion Arch Dermat Syph (Berlin) *194* 91–98 1952
10 *Got H* in Jadassohn s Handbuch der Haut und Ge chlechtskrankheiten Suppl IV/3 pp 99 100 Springer Verlag Berlin Göttingen Heidelberg 1962
11 *Kligman A* The bacteriology of normal skin In *Maibach H I & Hildick Smith G (ed) Skin bacteria and their role in infection* McGraw Hill Book Co New York Sydney Toronto London 1965 pp 13 31
12 *Marples M J* The ecology of human skin Charles C Thomas Publ Springfield Ill 1965 pp 669–670
13 *Peck S M & Hewitt W L* The production of an antibiotic substance similar to penicillin by pathogenic fungi Publ Hlth Rep *60* 148–193 1945
14 *Peck S M* Fungus antigens and their importance as sensitizers in the general population N Y Acad of Sci Ann *50* 1362 1375 1950
15 *Segretain G* *Infections by fungi that ordinary are saprophytes* Pulmonary aspergillosis Lab Invest *11* 1046–1052 1962
16 *Smith J M B & Marples M J* A natural reservoir of penicillin resistant strains of *Staphylococcus aur us* Nature *201* 844 1964
17 *Ur J* *Juh s P & Coban C* Über die Penicillinbildung von Trichophyton mentagr phytes Stammen Die Pharmazie *10* 709–713 19
18 *Uri J* *S athmary S & Herpay Zs* Über die Antibioticaproduktion von Pilzen am Ort ihre natürlichen Vorkommens Die Pharmazie *12* 485 488 1957

19 Wallerström A Production of antibiotics by *Epidermophyton floccosum* I The antibiotic spectrum of crude filtrate Acta path microbiol scandinav *71* 287–295 1967
20 Wright J M The production of antibiotics in soil I Production of gliotoxin by *Trichoderma viride* Ann appl Biol *41* 280–289 1954
21 Wright J M The production of antibiotics in soil III Production of gliotoxin in wheatstraw buried in soil Ann appl Biol *44* 461–466 1956

Acta path microbiol scandinav 74 543 560 1968

The Diagnostic Bacteriology Department (Chief H Lautrop M D)
Statens Seruminstitut Copenhagen Denmark

A NEW EPIDEMIC PHAGE TYPE OF *STAPHYLOCOCCUS AUREUS*

3 *Occurrence and Spread of 'Type 6557', with Special Reference to the Consumption of some Antibiotics*

By

P Bulow

Received 11 v 68

In two previous reports (*Bulow* 1968 a and b) it was mentioned that the epidemic occurrence of non typable staphylococcal strains called for the introduction of a new typing phage An experimental phage (6557) was found suitable as it was able to lyse the majority of multiple resistant non typable strains i e strains resistant to three or more of the antibiotics in general use

The present report attempts to describe the emergence and spread of the new kind of strains named type 6557 all over the country by using two materials registered at Statens Seruminstitut and previously referred to as bacteraemia material and "total material (*Bulow* 1968 b) A complete report of the latter has been published recently (*Rosendal & Bulow* 1967) while the bacteraemia material will be treated separately (*Rosendal et al* 1968)

Rosendal & Bulow (1967) found that the contribution of specimens isolated from boils and abscesses to the total material showed an absolute and a relative fall whereas the relative contribution of group II—and group III— strains (excluding the 834 6557 complex) was the same in 1961 and in 1965

Type 6557 staphylococci are now (1967) the most frequently isolated phage type from post operative wounds urine and faeces and the types belonging to the complex 834 6557 are the most common staphylococci in respiratory tracts

It has been noticed previously (*Rosendal et al* 1963) that the epidemically occurring types each had their special antibiogram and that that of type 6557 is characterized by including resistance to erythromycin neomycin and bacitracin (*Bulow* 1968 b) Therefore it was attempted to demonstrate a connection between the origin and spread

---

The author's present address Department of Medical Bacteriology Kommunehospitalet, Arhus C.

of the new phage type and the consumption of these antibiotics Two large hospitals were chosen for this survey as information about the consumption of antibiotics which was not available on a country wide scale could be obtained from the two local pharmacies

MATERIALS AND METHODS

From the total material only one strain (the first isolated) from each person has been registered

All strains from the bacteraemia material were accessible in lyophilized state

The Tween 80 reaction has been examined as described in Part 1 of this study

The consumption of antibiotics has been estimated on the basis of information given by the pharmacists at the two hospitals referred to in this investigation The antibiotics include penicillins (G and V penicillins semisynthetic penicillins among which the penicillinase resistant ones and a few others are included) streptomycins (streptomycin sulphate and dihydro streptomycin sulphate) tetracyclines (tetracycline-chloride oxytetracycline-chloride chlortetracycline chloride and dimethyl chlortetracycline-chloride) chloramphenicol (either the palmitate or the succinate) erythromycin (either as succinate lactobionate stearate propionyllaurylsulphate or glycoheptonate) neomycin and related compounds (kanamycin paromomycin and framycetin) all of which have been dispensed as sulphates and finally bacitracin

Information about the yearly turnover of patients was available in annual reports from each of the hospitals

RESULTS

Since April 1st 1963 the experimental phage 6557 has been used routinely together with the conventional typing phages and since then this type has continued to advance in hospitals all over the country While in 1963 7 per cent of all strains isolated (12 000 isolates) were lysed only by phage 6557 this number increased to 11 per cent in 1964 (14 400 isolates) and to 13 per cent in 1965 (14 800 isolates)

As type 6557 strains seem to have arisen from type 83A/6557 strains by lysogenization (*Jevons & Parker* 1964 *Bulow & Rosendal* 1964) the two types have been studied together

The spread of *Staphylococcus aureus* phage type 83A in Danish hospitals from 1957 to 1962 has been described by *Rosendal & Jessen* (1964)

Total Material

The staphylococcal situation in the hospitals all over the country during the last five years has been surveyed in a paper by *Rosendal & Bulow* (1967) Here it will be enough to deal with the following summary of the situation

It is seen from Fig 1 that the staphylococcal type situation in the hospitals of the whole country is changing from a clear dominance of group I isolates to a probably still increasing preponderance of group III isolates The increase of the latter group has been caused first by type 83A/6557 alone but later (since 1962) by the considerable rise in the number of type 6557 isolates (Fig 1 hatched area)

The decline of the curve representing group I is not a relative

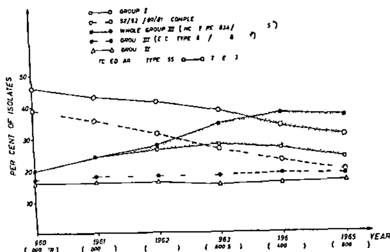


Fig 1

The staphylococcal situation in the whole country
(according to phage types or groups)

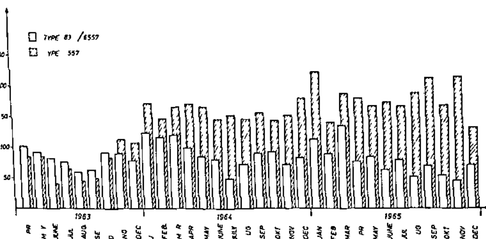


Fig 2

Occurrence of infections caused by staphylococci of the 83A 6557 complex
in the whole country

phenomenon only as the total number of isolated strains is falling and the tendency seems partly to be caused by a decreasing number of strains isolated from furunculi (boils) and abscesses (*Rosendal & Bulow* 1967)

The relative incidence of group II and group III (excluding 83A/6557 and 6557) is practically unchanged through the years 1960–1965 suggesting that the investigated materials are comparable from one year to another (i e the selection of samples is unchanged)

Fig 2 shows the number of the two related types 83A/6557 and 6557 from April 1963 and throughout the years 1964 and 1965 It appears that since the end of 1963 the number of strains of the new type has exceeded that of type 83A/6557 strains The number of type 83A/6557 strains seems to decline slowly during the last two years while the new type has shown a clear tendency to increase in number The seasonal variations which seem to occur in the number of isolated type 83A/6557 strains accumulating in the first months of each year do not seem to take place in case of the new type

Bacteraemia Material

The incidence of staphylococcal bacteraemia among patients with staphylococcal infections in all Danish hospitals during the years 1961–1965 according to the cases registered at Statens Seruminstitut appears from Table 1

TABLE 1

Incidence of Staphylococcal Bacteraemia According to the Centralized Registration at Statens Seruminstitut

Year	Number of bacteraemia cases	Number of patients with staphylococcal infections	Incidence of bacteraemia per 1000 cases of staphylococcal infections
1961	136	12 000	11.3
1962	180	12 400	14.5
1963	226	12 600	17.9
1964	250	14 400	17.6
1965	307	14 800	20.8

The table shows that the incidence of bacteraemia has undoubtedly increased (almost a doubling) during the five year period

The composition of the material according to phage types appears from Fig 3 in which the relative incidence of the various types or groups is indicated

It is seen that the variations in the relative composition of the yearly materials reflect the same situation as that shown in Fig 1 which represents the total material The 52 52A 80 81 complex is here divided into two groups of which the types 80 81 and 80/81 are markedly decreasing while 52/52A/80/81 (is one type) is fairly constant Type 83A/6557 reached its maximum in 1962 but since then the frequency of this type has decreased markedly

A certain proportion of the material has still been non typable (NT) but by retyping the material when new phages have been introduced it has been possible to trace such newly introduced phage types back to 1957 "Type 6557 or which seems to be more correct in this situation strains lysed by phage 6557 can be found among the bacteraemia strains registered primarily as NT since 1957

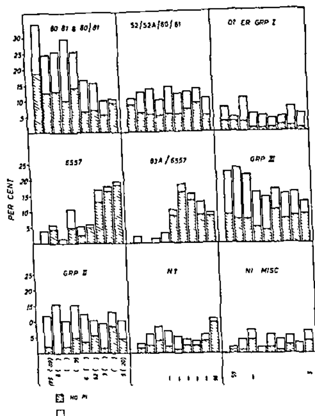


Fig 3
The composition of the bacteraemia material from the years 1957 to 1965 according to phage types or groups

It is surprising to find that about 11 per cent (14 out of 125) of strains from bacteraemia cases in 1960 were lysed only by phage 6557 From the preceding three years (1957–1959) 13 strains out of 372 (3 5 per cent) were lysed by the same phage only

Whereas the percentage of strains sensitive to all antibiotics or resistant only to P PS or PT in the material isolated from 1957 to 1961 was as much as 49 (17 out of 35) the percentage of strains with similar antibiotic sensitivity from the years 1962 to 1965 did not amount to more than 7 (11 out of 157) This will appear from Table 2 which gives a survey of antibiotic sensitivity and TW reaction of all type 6557 strains from bacteraemia cases during the years 1957–1965

It is seen that about 50 per cent of the strains from 1957–1961 were penicillin streptomycin and tetracycline resistant (PST) whereas only one was resistant to erythromycin too (TW +) and none to neomycin or bacitracin The rate TW—/TW + was 51 per cent (18/35)

These observations suggest that the strains lysed by phage 6557 from this early period may have composed a type of less resistance

TABLE 2

Survey of Antibiotic Sensitivity and TW Reaction of Strains of Phage Type 6557 from Bacteraemia Cases (total number)

Year(s)	TW	Sens	P	PS or PT	PST	PSTC	PSTNB	PSTE	PSTCE	PSTENB	PSTCENB
1957 to	+	2	8	3	3			1			
1961	—		3	1	14						
1962	+	2	1		1						
	—		1		2					4	
1963	+	2	1		3						
	—				6	1		3		18	5
1964	+		3		1						
	—				9	1	2	3	1	19	7
1965	+		1		6						1
	—				11	1		5	4	24	8

TW = Tween 80 reaction Sens = sensitive to all antibiotics Strains resistant to P = penicillin S = streptomycin T = tetracyclines C = chloramphenicol N = neomycin B = bacitracin E = erythromycin

= one strain resistant to methicillin
= two strains resistant to methicillin
= three strains resistant to methicillin

to antibiotics than is the case to day As staphylococci of that type are considered to have developed from type 83A staphylococci by lysogenization (*Jevons & Parker* 1964 *Bulow & Rosendal* 1964) and as the lysogenization as such does not cause a change in antibiotic resistance (*Jevons et al* 1966) one would expect the typical pattern of antibiotic resistance of type 6557 to be identical with that of type 83A/6557 i e PST (*Rosendal & Jessen* 1964 *Bulow* 1968b) and this seems really to be the case (Table 2)

However type 6557 strains did not prove to be of any particular importance as a cause of bacteraemia until the antibiotic pattern was extended to include erythromycin neomycin and bacitracin (PSTENB)

Sensitivity tests of all the bacteraemia strains (1957–1965) have revealed that the occurrence of the types 83A/6557 and 6557 is decisive for the general increase of the antibiotic resistance in this material

Fig 4 (left) shows that resistance to tetracyclines has been almost exponentially increasing since 1958 and that this increase can chiefly be ascribed to the two types mentioned It also appears from the figure (right) that type 6557 has been almost alone in enhancing the resistance to erythromycin and that the most distinct rise happened through the year 1963 More than 90 per cent of these erythromycin resistant strains from 1963–1965 are resistant to neomycin and bac-

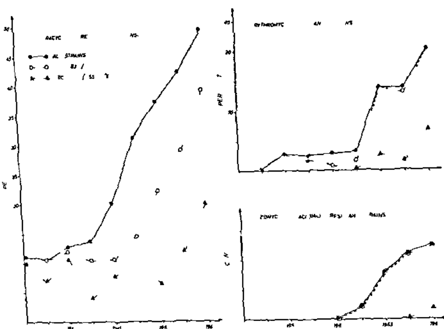


Fig 4

Incidence of tetracycline erythromycin and neomycin/bacitracin resistance among 1293 *Staphylococcus aureus* strains from cases of bacteraemia 1957–1965

tracin too whereas less than 2 per cent of other phage types (in 1965) are found resistant to the latter two antibiotics (namely the group III strains—insusceptible to typing phage 83A—mentioned in a previous paper (*Bulow* 1968b) and non typable strains (*Rosendal & Bulow* 1967)

Clinical Data

The new type has been chiefly confined to large hospitals with facilities for intensive therapy

Table 3 shows the relative incidence of some of the common staphylococcal groups or phage types in different hospital departments with various therapeutical functions The strains have been isolated (one from each patient) during 1964 and are registered according to their phage type It appears that type 6557 is isolated most frequently in departments with patients with lowered resistance to infections and subjected to intensive treatments

Strains of type 83A/6557 are most often isolated from patients in neurosurgical wards possibly because of the numerous tracheotomias in these departments and as reported previously (*Rosendal & Bulow* 1967) types 83A/6557 and 6557 are most often isolated from the respiratory tract

1526 strains of type 6557 isolated during 1963 and 1964 were forwarded to Statens Seruminstitut with satisfactory information about

TABLE 3

The Relative Incidence of Some Common Staphylococcal Phage Groups or Types in Hospital Wards with Different Therapeutical Functions
(All Strains were Isolated in 1964)

Number of strains	Isolated in (category of ward)	Phage type or group (percentage of strains) 80	III	834/6557	6557
318	Nephrology (peritoneal and hemodialysis)	10	13	7	31
348	Burns units	13	20	1	25
267	Thorax surgery	20	17	9	22
952	Common surgery	23	20	5	16
311	Neuro surgery	13	19	14	15
395	Internal medicine	13	24	8	15
203	Radiology	22	28	5	11
152	Far-nose-throat	18	20	3	7

80 = 52 52A 80 81 complex
III = lysed by one or more the group III phages

the sources from which they were isolated They were arranged according to sources and compared with some other phage types The distribution of strains of the new type expressed as percentages is compared with the distribution of type 83A/65o7 (1510 strains) the 52 52A 80 81 complex (3888 strains) and strains from group III (3383 strains) excluding types 83A/65o7 and 6557

The results of this comparison are given in Fig 5

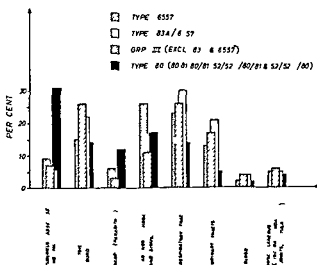


Fig 5
The relative distribution of some staphylococcal phage types (1963 and 1964) from different sources

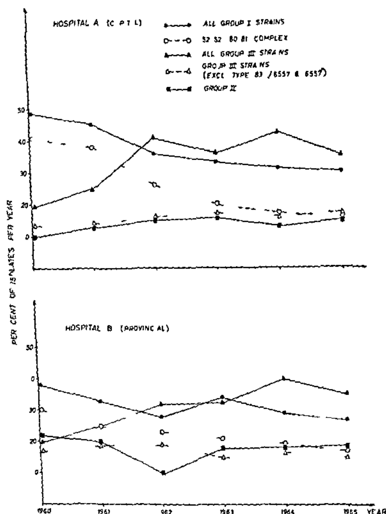


Fig 6

The staphylococcal situation in two Danish hospitals
(according to phage types or groups)

As seen from this figure the new type is *most often isolated from* cicatrices and wounds respiratory and urinary tracts while it is more rarely isolated from primary cutaneous processes (furunculi mastitis paronychia etc) In this respect it resembles type 83A/6557 whereas the 52 52A 80 81 complex behaves in the opposite way

Epidemic Spread in Two Separate Hospitals

Type 6557 staphylococci seem especially to have advanced in hospitals where type 83A/6557 was previously known to be wide spread and such a situation found in two separate hospitals one in the

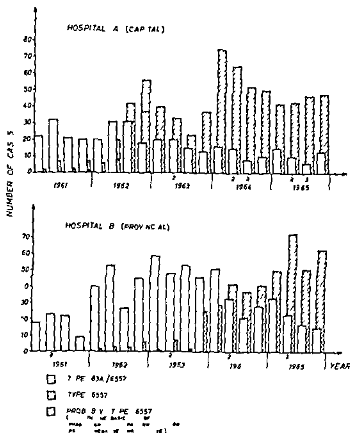


Fig. 7

Occurrence of infections caused by staphylococci of the 83A 6557 complex in two Danish hospitals (given for three month periods)

capital (hospital A) and one in a provincial town (hospital B). These hospitals of about the same size (1000 beds) were chosen as representative of the hospital situation in the whole country.

The general situation in the two hospitals is illustrated in Fig. 6 showing the relative incidence per year from 1960 to 1965 of some of the most important groups or types of *Staphylococcus aureus*.

The variations in the staphylococcal population in both hospitals correspond to those of the total material shown in Fig. 1. However, in hospital A the changes are more pronounced than those found in hospital B as well as in the total material.

The decreasing relative number of staphylococcal isolates of type 83A/6557 and 6557 through 1965 is due to a decreasing total number of type 83A/6557 isolates (see Fig. 7) and also to an increasing number of NT strains in the material.

Fig. 7 shows the number of registered cases of staphylococcal infections caused by strains of types 83A/6557 and 6557 in the two

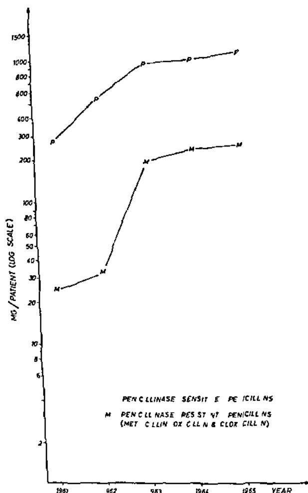


Fig 8

The use of penicillins (some hospitals in Copenhagen including hospital A (3700 beds))

hospitals (A and B) The number of cases is counted and indicated in three month periods

An attempt has been made to find out when the new type began to emerge as hospital A was the first hospital in which the multiple resistant non typable strains were isolated Some of the early strains have been preserved and later tested for sensitivity to phage 6₀57 Other strains have not been available for retyping but were registered as NT with information about antibiotic pattern sensitivity to mercuric chloride and TW reaction Such NT strains resistant to penicillin streptomycin and tetracycline and most of them to erythromycin (i e the patterns PST or more often PSTE) mercury resistant and TW negative are indicated in Fig 7 as probably type 6₀57

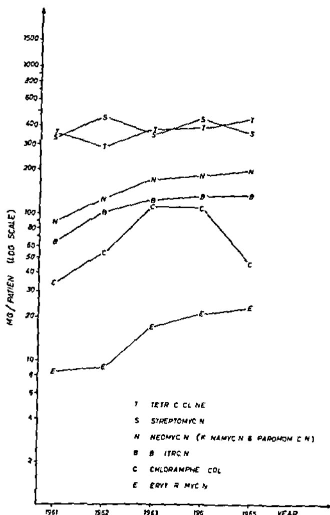


Fig 9

The use of antibiotics (some hospitals in Copenhagen including hospital A (3700 beds))

In hospital A a few sporadic cases of infection caused by type 6557 occurred during 1961 whereas a real epidemic accumulation of the type has been registered during 1962 and since then type 6557 has surpassed type 83A/6557 It is seen that the number of type 83A/6557 isolates has decreased slowly since 1962 while type 6557 has increased considerably

In hospital B an accumulation of staphylococcal infections caused by type 83A/6557 was noticed during the years 1962 and 1963 and as seen from Fig 7 a few sporadic cases caused by "type 6557 may have occurred in 1962 However it was not until the last three months of 1963 that a real accumulation of such cases seems to have taken place This accumulation continued during 1964 and the number of "type

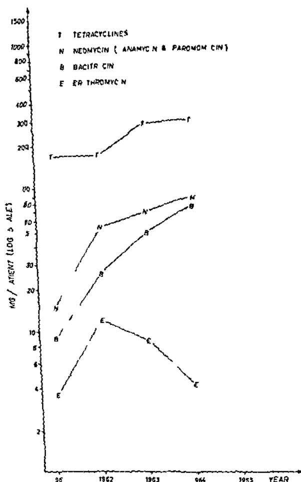


Fig 10

The use of some antibiotics (provincial hospital B about 1000 beds)

6557 cases exceeded that of type 83A/6557 which since then decreased in time with the increase of the number of cases caused by the new type So the emergence and spread of the new type seem to "depress type 83A/6557

The Consumption of Antibiotics

Hospital A in the capital contributes by about 1000 beds to the area of supply from one pharmacy which serves hospitals with a total capacity of 3700 beds It has a yearly turnover of patients of about half that of the whole area (viz 36 000 patients per year) and is thus the most therapeutically active hospital among those within the area

Hospital B is as mentioned before of about the same size and capacity as hospital A so they seem to be comparable

The antibiotics used by the provincial hospitals B comprise tetracyclines erythromycin neomycin and related compounds and bacitracin whereas information about the consumption of penicillins and streptomycin was not available

Figs 8 and 9 illustrate the antibiotic consumption in hospitals in Copenhagen The use of penicillins was increasing during the five years 1961 to 1965 and the most vigorous increase is seen through 1961 to 1963 It is seen that the consumption of methicillin and related (penicillinase resistant) penicillins has grown ten fold while the increase is more moderate (about four fold) as regards the other penicillins among which the classical G and V penicillins greatly predominate

The use of streptomycins and tetracyclines was almost equal and remained fairly constant throughout the five years (300–400 mg per patient per year) and corresponds to that found by *Lund* (1956) in the same hospitals in 1952

Neomycin and bacitracin are very often used in combinations so the curves run parallel and are increasing two fold again the biggest rise seems to occur between 1961 and 1963

The use of chloramphenicol has increased two to three fold during 1961–1963 but since then the tendency seems—for unknown reasons—to be falling

Erythromycin seems to have been used in considerably less amounts than the other antibiotics even though a doubling of the consumption during 1962–1965 is noticed

In the provincial hospital B the use of erythromycin neomycin and bacitracin has on the whole been less than in hospitals in the capital Fig 10 shows that the use of tetracyclines was about 200–300 mg per patient per year The use of neomycin and bacitracin has increased six and eight fold respectively during the four year period 1961 to 1964 A comparison of Fig 9 and Fig 10 shows that it was not until 1964 that the consumption of neomycin and bacitracin in the provincial hospital B reached the same high level as that seen in hospital A already in 1961–1962

The peak (in 1962) on the curve showing the use of erythromycin may be due to the intensive treatment of a few patients but on the whole the use of this antibiotic was very slight

Other macrolide antibiotics (*e g* oleandomycin spiramycin and carbomycin) have not been used in any of the hospitals

By investigation of the proportions of antibiotics used for local and systemic treatment it was found that only the penicillins and streptomycins have been used systemically only 2 per cent of erythromycin was used locally (eye ointment) while 10 15 per cent of tetracyclines and chloramphenicol was dispensed as ointments and drops The tetracyclines for local use were often combined with adrenal steroid derivatives Sprays containing this combination were used but in small amounts

Neomycin and bacitracin were as a whole not administered systemically 50 to 60 per cent was dispensed as tablets for so called preoperative intestinal disinfection 40 to 50 per cent was dispensed as powders ointments solutions and a remarkably large amount of the combined preparation was used as sprays (*e g* 1000 sprays per year (250 mg neomycin + 125 mg bacitracin per spray) in one hospital with 1000 beds) The last two antibiotics were also very often administered in combination with adrenal steroid derivatives

DISCUSSION

In the present report it was found that the fluctuations of the staphylococcal population in two big hospitals corresponded to those found in the material from the whole country but that the appearance of the new multiple resistant type 6557 in the provincial hospital was delayed for about two years

Several authors have discussed the theory of a connection between the consumption of antibiotics and development of antibiotic resistance in staphylococci *Lepper et al* (1953) *Wise et al* (1955) and *Bauer et al* (1960) have found a positive correlation whereas *Schneierson* (1955) *Petersdorf et al* (1960) and *Altemeier* (1957) have not been able to find any connection at all *Waisbrenn & Strelitzer* (1958) agreeing with the latter authors put forward the interesting theory that the sudden spread of penicillin resistance among *Staphylococcus aureus* strains might be compared with the rapid world wide mutation of influenza virus providing an example of a species change that occurred without a known human influence (*Burnet* 1953) This theory has within recent years been made plausible by the demonstration that transducible extrachromosomal plasmids might control penicillinase production by staphylococci (*Novick* 1963 *Harmon & Baldwin* 1964 *Richmond* 1965)

In trials to correlate the appearance of the new epidemic type in the two Danish hospitals with the consumption of neomycin bacitracin and erythromycin one finds that the spread of the type coincides with the time when the use of neomycin (and bacitracin) reaches the same high level in both hospitals *i e* later in the provincial hospital than in the hospital in the capital Erythromycin however is used to almost the same extent at the same time in both hospitals

Theoretically antibiotic resistance in bacteria can originate as a result of stepwise mutation in connection with a selective pressure or it may be due to transfer of genetic material The latter mechanism seems the more probable as regards erythromycin resistance in neomycin resistant strains belonging to type 6557 since erythromycin resistance found in these strains belongs to the inducible type (*Bulow* 1968b) whereas erythromycin resistance obtained by selective proce

dures has invariably been of the constitutive type (*Haight & Finland* 1952 *Jones et al* 1956)

As to neomycin resistance the findings reported leave open the possibility that it may be due to stepwise mutation under the condition of a selective pressure especially since neomycin has often been dispensed in a way (spray etc) which increases its area of action It is permissible to suggest that a further spread of the neomycin resistant strains may be caused by transduction as virus epidemics (bacteriophages) from time to time may attack bacterial populations in hospitals This kind of genetic transfer may create a bacterial population more capable of resisting the high selective pressure existing in modern hospitals

Jacobs & Willis (1964) found it unlikely that the emergence of neomycin resistant staphylococci was associated with exposure to this or related antibiotics The authors were unable to isolate neomycin resistant strains that were tetracycline sensitive and they suggested the existence of some sort of connexion between the resistance to these two antibiotics They made it probable that there is a group of staphylococci that is inherently resistant to neomycin or that agencies other than exposure to neomycin have been responsible for the emergence of strains resistant to its action

It is probable that the genetic markers for resistance to various antibiotics (*e g* T I N B) are linked (*Bulow* 1968b) and it is obvious that bacteria in hospital environments receiving more than one marker at the same time (from an originally resistant individual) will have an advantage over those receiving only one Thus the comparison with the influenza virus pandemics (*Waisbrenn & Strelitzer* 1958) does not seem quite implausible

The origin of the new phage type certainly is phage dependent as it has been shown in England and Denmark that it has developed from the previously epidemically occurring type 83A by lysogenization (*Jevons & Parker* 1964 *Bulow & Rosendal* 1964)

The new types belonging to the 83A 6557 complex differ from the previous epidemic types of the 52 52A 80 81 complex (type 80) as to the kind of clinical infection they cause and as to the category of *hospital departments in which they are most often isolated*

The biochemical background of the nosological specificity will be discussed later here it is pointed out only that whereas the typical "type 80 infection was boils etc the new types are characterized by their affinity for the respiratory and urinary tracts (Fig 1) and for post-operative wounds These properties may have caused them to spread fairly easily in some hospital departments (Table 3)

Thus the three criteria mentioned by *Williams* (1962) with a view to describing strains as epidemic 1) a certain degree of antibiotic resistance 2) ability to invade and cause disease and 3) ability to spread seem to apply to the new types

SUMMARY

A survey of prevalent staphylococcal phage types in Denmark during the years 1960-1965 shows that a new type lysed by the experimental phage 6557 has gradually increased in frequency and is now the most common epidemic type

The type is resistant to several antibiotics including tetracycline erythromycin neomycin and bacitracin

Its spread in two hospitals studied in detail coincides with a high level of neomycin and bacitracin consumption but seems unrelated to the use of erythromycin

Since erythromycin resistance in these strains hardly can be a result of a selection it is assumed that the emergence and spread of this particular phage type is unrelated to a high erythromycin consumption Resistance to erythromycin might be explained by the existence of a genetic linkage between resistance to this antibiotic and resistance to neomycin (and bacitracin) and/or possibly to other antibiotics

Type 6557 (and 83A/6557) has occurred mainly in intensive care units and has most frequently been isolated from the respiratory tract post operative wounds urinary tract and blood

REFERENCES

Altemeier W A Cyclic variations in the resistance in staphylococci isolated from surgical infections during the past five years Antibiot Ann p 6?9–633 1957 (N Y Med Encyclopedia Inc 1957)

Bauer A W Perry D M & Kirby W M M Drug usage and antibiotic susceptibility of staphylococci J Amer Med Ass *173* 475–480 1960

Burnet M The influenza virus Scientific American *188* 27–31 1953

Bulow P A new epidemic phage type of *Staphylococcus aureus* 1 The experimental typing phage 6557 Acta path microbiol scandinav 7? 147 159 1968a

Bulow P A new epidemic phage type of *Staphylococcus aureus* ? Characteristics of staphylococci lysed by phage 6557 Acta path micr obiol scandinav 72 160–175 1968b

Bulow P & Rosendal K Epidemic spread of a new type of *Staphyloc ccus aureus* Proc XIV Scand Congr Path Microbiol Oslo p ?30–?32 1964 (Norweg Univ Press Oslo)

Harmon S & Baldwin J N Nature of the det rminant controlling penicillin[illegible] duction in *Staphylococcus aureus* J Ba t *87* 593–597 1964

Haight T H & Finland M Resistance of bacteria to erythromycin Pr c [illegible] Biol Med *81* 183–188 195?

Ja obs S I & Willis A T Some phy iologi al characteristics of [illegible] and kanamycin resistant strains of *Staphyl coccus aureus* J clin [illegible] 616 1964

Jevons M P John M & Parker M T Cultural cha acters of a [illegible] group of hospital staphylo oc i J cl n Path *19* 305–312 19[illegible]

Jev ns M P & Parker M T The e olut on of new hosp tal [illegible] coccus aureus J clin Path *17* 243–250 1964

Jones W F jr Nichols R L & Finland M Development of [illegible] esistance in vitro t erythromycin bomycin spi mycin [illegible] st eptogramin P o S c Fxp Biol Med 93 388–393 19[illegible]

Lepper M H Dowl ng H F Jackson G C Moulton B & [illegible] antibiotic usage in the hospital on the in dence of [illegible] among personn l carrying taphylococci J Lab clin [illegible] 1953

Lund F Forekomsten of kemoresistente stafylokokker på hospitalsafdelinger Thesis p 134 (Dansk Vidensk Forl Kbh) 1956

Novick R P Analysis by transduction of multations affecting penicillinase formation in *Staphylococcus aureus* J gen Microbiol 33 121–136 1963

Petersdorff R C Rose M C Minchew H B Keene W R & Bennett I I The sensitivity of hemolytic staphylococci to a series of antibiotics II A three year progress report Arch intern Med 105 398–412 1960

Richmond M H Dominance of the inducible state in strain of *Staphylococcus aureus* containing two distinct penicillinase plasmid J Bact 90 370–374 1965

Rosendal K Personal communication

Rosendal K & Bulow P *Staphylococcus aureus strains isolated in Danish hospitals* 2 From January 1st 1961 to December 31st 1965 Acta path microbiol scandinav 71 422–438 1967

Rosendal K Bulow P Eriksen K R Faber V & Jessen O Changing staphylococci 1 A ten year study of bacteraemia strains 1968 (To be published)

Rosendal K & Jessen O Epidemic spread of *Staphylococcus aureus* phage type 83A Acta path microbiol scandinav 60 571–576 1964

Rosendal K Stenderup A Helms I & Eriksen K R *Staphylococcus aureus* strains isolated in Danish hospitals from April 1st to December 31st 1960 Acta path microbiol scandinav 58 72–84 1963

Schneirson S S Comparative sensitivity to penicillin and erythromycin of 505 strains of *Micrococcus pyogenes var aureus* isolated during 1953 and 1954 Antibiot Med 1 442–445 1955

Waisbren B A & Strelitzer C L A 5 year study of the antibiotic sensitivities and cross resistances of staphylococci in a general hospital Antibiot Ann p 350–364 1958 (N Y Med Encyclopedia Inc 1958)

Williams R E O Ecological evidence for the existence of virulent strains of staphylococci Recent Progress in Microbiology VIII Int Congr Microbiol (Montreal 1962) p 551–560 1962

Wise I Voigt A E Collin M V & Granny C L Origin of erythromycin resistant strains of *Micrococcus pyogenes* in infections Bacteriophage types and in vitro resistance of cultures to antibiotics Arch intern Med 9 419 426 1955

Acta path microbiol scandinav 74 551-557 1968

The Diagnostic Bacteriology Department (Chief H Lautrop MD)
Statens Seruminstitut Copenhagen Denmark

A NEW EPIDEMIC PHAGE TYPE OF *STAPHYLOCOCCUS AUREUS*

4 *Pathogenicity of Type 83A/6557 and Type 6557*

By

P BULOW

Received 11 i 68

A special aspect of the pathogenicity of *Staphylococcus aureus* will be dealt with in this paper relating to the ability to cause a fatal outcome in defined types of infection *e g* bacteraemia

Several examples of a correlation between phage type and the ability to cause certain sorts of lesions have been recorded Impetigo is often caused by type 71 or related types of group II (*Barrow* 1955 *Parker et al* 19__ *Smittlehouse* 1955 *Schmidt et al* 1957) Group I strains have been isolated preferentially from boils and wound infections but more rarely from the respiratory tract urine and stools (*Rosendal & Bulow* 1967) The latter sources often yield group III strains and these strains furthermore have almost equalled group I strains as causes of wound sepsis (*Walmark & Finland* 1961 *Rosendal et al* 1963) Group III also includes the enterotoxin producers (*Gillespie* 1947 *Allison* 1949 *Allison et al* 1949)

Type 83A strains have been isolated with high frequency from sputum (*Martin* 1963) and blood cultures (*Rosendal & Jessen* 1964)

The newer types lysed by one or more of the experimental phages B5 77Ad UC18 D or SF (*Comtois* 1963 1965 *Rountree & Beard* 1965) or characterized by resistance to neomycin and inhibition reactions with group III phages (*Mitchell* 1964) have occurred particularly in operation wounds burns urine and sputum but rarely in blood cultures (*Cohen et al* 1962 *Rountree & Beard* 1965) In recent years they have caused many cases of enterocolitis in the U S A (*Finegold & Gaylor* 1960 *Tisdale et al* 1960 *Hummel et al* 1963 *Altemeier et al* 1963 *Lavine et al* 1965) and enterotoxin of type S 6 has been demonstrated in strains lysed by phage UC18 (*Altemeier et al* 1965)

In Denmark strains of the types 83A/6557 and 6557 have likewise been isolated commonly from wounds urine and the respiratory tract but a feature quite distinct from the above mentioned experiences

The author s present address Dept of Medical Bacteriology Kommunehospitalet Aarhus C Denmark

these types are also of outstanding importance as causes of bacteraemia In previous large Danish materials (*Jessen et al* 1963) the prevalent bacteraemia types have reflected the composition of the general staphylococcal population in hospitals but the new types are heavily over represented in bacteraemia cases where type 6[illegible]7 was the most common single type in 1965

The following report gives details of the occurrence of the types 83A/6[illegible]7 and 6[illegible]7 in bacteraemia and of the mortality in such cases

MATERIAL

The material of bacteraemia strains and the total material representing the general staphylococcal population in Danish hospitals were described in part 2 of this study (*Bülow* 1965b)

Clinical information about the cases was provided by hospital departments concerned

RESULTS

Frequency of Types 83A/6[illegible]7 and 6[illegible]7 in Bacteraemia

In agreement with earlier findings (*Jessen et al* 1963) the type complex 52 52A 80 81 and group III exclusive of types 83A/6[illegible]7 and 6[illegible]7 are still found in the same proportion in bacteraemia strains and in the total material representing the prevalent staphylococcal population whereas group II strains may be somewhat under represented in the bacteraemia material

However types 83A/6[illegible]7 and 6[illegible]7 are found in bacteraemia material about twice as frequently as in the total material and the percentage of strains of these types resistant to four (or more) antibiotics is higher in bacteraemia material

TABLE 1

The Representation (per cent) of Some Phage Types and Groups in the Total Material and in the Bacteraemia Material from the Years 1963 and 196[illegible]

	19[illegible]3		1964	
	Total (1[illegible] 600)	Bacteraemia ([illegible])	Total (14 400)	Bacteraemia ([illegible])
[illegible] 52A 80 81 complex	[illegible]	[illegible]	[illegible]	[illegible]
Group II	14	[illegible]	15	[illegible]
Group III (excluding the 83A [illegible] complex)	18	[illegible]	19	16
Type 83A ([illegible])	3	[illegible]	7	[illegible]
*Type 6[illegible]		17	15	18

[illegible] — standard error of the difference [illegible] ($p < 0.01$)
Total number of strains in brackets

TABLE 2

Percentage of "Type 6557" Strains in the Total Material as Compared with the Bacteraemia Material According to Their Antibiotic Resistance

Resistant to the antibiotics	1963 Total (8 ?00)	1963 Bacteraemia (73?)	1964 Total (12 800)	1964 Bacteraemia (255)
P S and T (including P C, N B)	5	16	9	17
P S T and E (including C N B)	3	11	5	12

Total number of strains in brackets
Number of strains from the total material examined for antibiotic resistance

Mortality in Bacteraemia Cases

Cases due to type 83A/6557 and to "type 6557" were compared with cases due to the classic epidemic strains of "type 80" taken as the type complex 52 52A 80 81 However these three categories were not directly comparable owing to differences in age of the patients origin of the infections and pre existing clinical complications three factors known to influence lethality

Age distribution (Fig 1) The number of patients below the age of 40 is higher in "type 80" infections (?7 per cent) than in infections with type 83A/6557 (11 per cent) and "type 6557" (13 per cent)

Origin of infection Hospital acquired infections were somewhat less common in cases due to "type 80" than in cases due to the other two types The proportions of cases ascribed to hospital infection were

52 52A 80 81 complex	65 per cent (109/167)
type 83A/6557	83 per cent (83/100)
type 6557	86 per cent (76/88)

Pre existing complications Lethality as well as susceptibility to bacteraemia in fection is augmented by diseases such as malignant tumours severe blood diseases diabetes cirrhosis of the liver ulcerative colitis skin diseases and treatment with steroids or cytotoxic agents

If limited to hospital infections in patients more than 40 years old the materials were comparable as to the occurrence of these complications which were present in about 40 per cent of the patients of each of the three categories

The list of predisposing conditions does not include severe renal failure since it was impossible to distinguish between renal failure as a consequence of the infection and as a predisposing condition Comments on this problem are given below

Comparable and reasonably large groups were established by limiting the material to patients more than 40 years old infected in hospital and not suffering from any of the complicating conditions listed above

Mortality rates for infections due to types 83A/6557 and "6557" are practically identical and about twice the mortality rate in "type 80" infections (Table 3) In the following the first two categories will be considered together

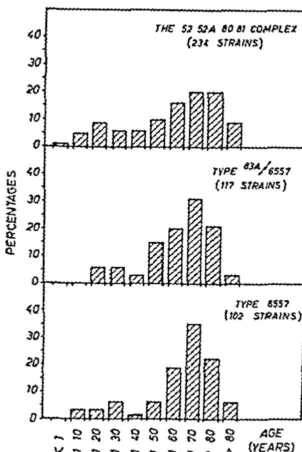


Fig. 1

Age distribution of patients with bacteraemia due to different phage types (1961–1964)

TABLE 4

Comparison of Mortality Rates in Cases of Bacteraemia (1961–1964) Due to Staphylococci of Different Phage Types

Phage type	Number of cases	Mortality rate (per cent)
52/52A/80/81 complex	67	37
Type 83A/6557	49	68
Type 6557	44	66

In 78 of the 86 fatal cases full details of the clinical course could be obtained. An analysis of these cases (Table 4) showed that the difference in mortality was unrelated to any difference in mean age or in the more or less acute course of the infection, but in the patients infected with the new types uraemia or anuria was remarkably common

However the figure of 28 per cent as compared with 13 per cent in type 80 infections (in Table 4) is not statistically significant (P_1–P = 15 per cent E_D = 9 2 per cent)

If patients with these renal complications are excluded the mortality rates for the groups approach each other although the differences still are statistically significant (Table 5) The figures representing the true mortality due to the staphylococcal infection probably lie between the figures in Table 3 and those in Table 5 because it must be assumed that renal failure was a predisposing complication in some cases whereas in others it was a result of the infection

TABLE 4

Comparison of 78 Fatal Cases of Bacteraemia

	Caused by the type complexes	
	52 52A 80 81	83A 6557
Number of deaths	24	54
Average age at death	71 years	66 years
Death within 1 week	60 %	63%
Death during 2 4 weeks	22%	24%
Death later	18%	13
Uraemia or anuria present	13%	28%
	(= 3 patients)	(= 15 patients)

TABLE 5

Comparison of Mortality Rates in Cases of Bacteraemia after Exclusion of Patients with Renal Failure (1961–1963)

Phage type	Anuria/uraemia cases excluded		Anuria/uraemia and chronic pyelonephritis cases excluded	
	Number	Mortality (%)	Number	Mortality (%)
52 52A 80 81 complex	60		52	31
83A/6557	38	[illegible]	36	[illegible]
6557	31		29	

Another factor excluded from [illegible] list of factors [illegible] the infection is the surgical proc[illegible] preceding the [illegible] Th[illegible] measure of such procedures [illegible] self influence th[illegible] condition of the patients it may [illegible] the severity of [illegible] order and the procedures [illegible] conditions for [illegible] infections foci The degree of su[illegible] ocedures was rat[illegible] index is outlined below

For each patient th[illegible] surg[illegible] ere e aluated a below and the total of the p[illegible] h patient as taken as th[illegible]

1 point Intra en[illegible] infu[illegible] n pun[illegible] ture of [illegible] cision and excision m[illegible]

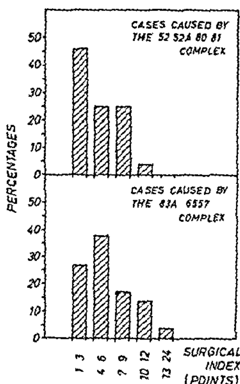


Fig 2

Surgical index in patients with fatal bacteraemia due to strains of the 83A 6557 complex as compared with the 52 52A 80 81 complex

2 points Venous cut down, tracheostomy, transurethral prostatic resection

3 points Minor abdominal operations (explorative laparotomia, appendectomia, herniotomia), transvaginal gynaecological operations, catheterization of caval vein

4 points Major abdominal operations (e g on the biliary or gastro intestinal tracts), osteosyntheses, major amputations, transvesical prostatectomy, secondary suturing and revision of ruptured surgical wounds, prolonged drainage of various cavities, renal operations

5 points Thoracotomy, extracorporal circulation, extracorporal and peritoneal dialysis

An arithmetical mean of the surgical indices is not relevant because of a large spread. A graphical representation of the results is given in Fig 2 which seems to indicate a certain preponderance of the extreme surgical intervention in patients infected with types 83A/6557 and 6557.

Even if such cases, i e with indices from 10 to 24 are excluded, lethality is significantly higher in infections due to the new types (63 per cent) than in those due to type 80 (40 per cent).

DISCUSSION

The principal results pertaining to the pathogenicity of the types 83A/6557 and 6557 may be summarized as follows

1) They seldom cause boils and other infections of intact skin in contrast to types of the complex 52 52A 80 81

2) They cause bacteraemic infections far more frequently than might be expected from their relative representation in the general hospital staphylococcal population

3) Mortality in bacteraemia cases exceeds mortality due to bacteraemia caused by strains of the complex 52 52A 80 81

4) The strains are on the whole characterized by the negative Tween reaction the antibiotic patterns PST and PSTI NB (*Bulow* 1968a and b) in contrast to the pattern PS characteristic of type 80 (*Rosendal & Bulow* 1967) and a high hyaluronidase production (*Faber Bulow & Rosendal* to be published)

The first point is compatible with the statements by other authors (*Rountree & Beard* 1965 *Comtois* 1965 *Lavine et al* 1965) that the very similar Australian and North American new type 83/77Ad was unable to infect intact skin This characteristic will modify the mode of spread of infections and the clinical character of infections as compared with *e g* the type complex 52 52A 80 81 known as a common cause of boils and paronychia (*Bulow* 1968c)

In a search for the bacterial basis for the ability to cause certain lesions it is natural to consider three properties that distinguish the types 83A/6557 and 6557 from the complex 52 52A 80 81 *viz* lack of the Tween 80 splitting enzyme multiple resistance (PST at least) and a high hyaluronidase production of the first two types

Lack of the ability to produce Tween 80 splitting enzyme seems correlated to a high mortality rate (*Rosendal* 1962 *Jessen et al* 1963 *Jessen et al* 1968) The rate was also found to increase with increasing multiple resistance of the staphylococci

A comparison between bacteraemia cases caused by epidemic strains with the same antibiotic resistance (*e g* PST) belonging to different phage types (including the new ones) showed that the mortality rate seemed to be unrelated to the susceptibility to the typing phages (*Jessen et al* To be published)

From the figures reported in this study one may conclude that the new types are highly virulent by virtue of their tendency to establish bacteraemia with a high mortality One of the possible sources of error in such a conclusion is that the new types may be endemic in departments treating special categories of patients with serious predisposing conditions (*Bulow* 1968c) But even when such patients (*Farrer & McLeod* 1960 *Minchew & Cluff* 1961 *Cohen et al* 1964) or patients undergoing extensive surgical operations (*Cohen et al* 1964) are excluded mortality rates for infections with the newer types exceed the rates for infections with *e g* strains of the 52 52A 80 81 complex which is considered highly virulent (*Williams* 1958 *Fekety & Bennett* 1959 *Williams* 1962)

A discussion of virulence is complicated by the fact that the anti

TABLE 6

Mortality Rate of Staphylococcal Bacteraemia According to Literature

Authors	Year	Number of cases	Mortality rate (%)	Special remarks
Lowenstein	1931	18	[illegible]	
Neuhof et al	1934	44	66	
Dolman	1934	64	75	Treated with antitoxic serum
Scott	1935	[illegible]	79	
McNeal & Frisbee	1936	100	73	
Rosenow & Brown	1938	29	66	
Scarpellino	1939	187	91	
Mandell	1939	35	83	
Skinner & Keefer	1941	122	82	
Harrell & Brown	1941	44	63	
Keefer et al	1943	91	40	
Anderson	1944	550	29	
Spink & Hall	1945	28	29	
Knight & Collins	1955	8	25	
Spink	1956	41	80	
Finland & Jones	1956	196	45	
Wilson & Hamburger	1957	55	[illegible]	
Tze Ying & Sehu Chen	1957	116	41	
Schirger et al	1957	109	34	Endocarditis excluded
Hassall & Rountree	1959	86	59	
Waisbren & Abboud	1960	100	64	
Hay	1960	71	50	
Faber et al	1960	201	41	
Powell	1961	49	82	
Sibon	1962	[illegible]	14	All treated with methicillin
Allen et al	1962	22	27	All treated with methicillin
Jessen et al	1963	462	39	
Kucers & Bennett	1964	33	36	Non surgical bacteraemia
Jensen	1967	43	23	41 cases treated with methicillin

The lines separates the pre antibiotic from the antibiotic era

biotic resistance of the strains influences the outcome of the infections (*Shooter et al* 1958, *Barber et al* 1960, *Jessen et al* 1963) owing either to a correlation between antibiotic resistance and certain virulence factors or to the dependence between therapeutic possibilities and antibiotic resistance. An elucidation of this question would require a material of untreated cases or cases treated with antibiotics for which all strains were invariably sensitive.

In the pre antibiotic era the literature tabulated in Table 6 shows a mortality of about 70 80 per cent declining after the introduction of penicillin in 1940, but no further decline is seen after the introduction in the early fifties of the bacteriostatic antibiotics chloramphenicol, tetracyclines and erythromycin. On the contrary the increasing antibiotic resistance seems to parallel an increase in mortality, and in 1961 *Powell* concluded that staphylococcal septicaemia has become as lethal a hazard as it was prior to the advent of antibiotics.

But again we are left with three possible explanations of the high mortality in bacteraemia caused by the new types approximating that of untreated cases from the pre antibiotic period (1) *resistance to an increasing number of antibiotics* (2) enhanced virulence or (3) altered host factors *e g* higher age use of extensive surgery etc

Still it seems that a sufficiently effective antibiotic administration is able to overcome a possible increase in virulence of the strains since for instance the introduction of methicillin reduced mortality (*Allen et al* 1962 *Siboni* 1962 *Jensen* 1967) to the same low level as did the introduction of penicillin 20 years earlier (*Barber* 1965)

The difference in hyaluronidase production of the types 83A/6557 and 6557 on the one hand and the 52 52A 80 81 complex on the other which probably influences the tendency of these types to cause skin infections *(Jessen & Bulow* to be published) may be significant also for their ability to cause bacteraemia infections in accordance with the concept of hyaluronidase as a spreading factor (*Duran Reynals* 1942) promoting bacterial invasiveness

It appears that various aspects of pathogenicity may differ independently and that staphylococci of different phage types may differ so considerably in their properties that they are comparable with for instance different serotypes of *E coli*

It may be concluded then that staphylococci of the new type complex and particularly the new epidemic strain 6557 occurring in the present hospital milieu with its large number of susceptible individuals are highly virulent since they are able (1) to spread (2) to invade and (3) to contribute to the death of their hosts

SUMMARY

In a material of strains from staphylococ[illegible]al bacteraemia the types 83A/6557 and 6557 are far more [illegible] represented than the generally occurring staphylococcal population in hospitals and mortality in these cases exceeds mortalit[illegible] s for the previously dominating type complex 52 52A 80 81 [illegible] influencing the [illegible] of the infections and the compar[illegible] of different [illegible] are discussed

The relation between clin[illegible] and th[illegible]gical properties of the new types is [illegible] with spec[illegible] to their multiple antibiotic resist[illegible] Tween 80 [illegible] and high hyaluronidase production

It is concluded that th[illegible] types are highly [illegible] in the present hospital environm[illegible]nt

REFERENCES

Allen J D Robert[illegible] F [illegible] by [illegible] M M Staphylococcal sept[illegible] treated with methi[illegible]llin Report of [illegible] cases Ne[illegible] Engl J Med [illegible]

Anderson D C Clinical experience with penicillin N Y State J Med 44 1651-1654 1944

Allison V D Food poisoning Proc roy Soc Med 42 214-220 1949

Allison V D Hobbs B C & Martin P H A widespread outbreak of staphylococcal food poisoning Mth Bull Minist Hlth Lab Serv 8 38-47 1949

Altemeier W A Hummel R P & Hill E O Staphylococcal enterocolitis following antibiotic therapy Ann Surg 157 847-858 1963

Barber M Hospital staphylococci and the new penicillins Minerva med 56 Suppl 3058-3059 1965

Barber M Dutton A A C Beard M A Elmes P & Williams R Reversal of antibiotic resistance in hospital staphylococcal infection Brit med J 1 11 17 1960

Barrow G I Clinical and bacteriological aspects of impetigo contagiosa J Hyg (Lond) 53 495 508 1955

Bülow P A new epidemic phage type of *Staphylococcus aureus* 1 The experimental typing phage 6557 Acta path microbiol scandinav 73 147-159 1968a

Bülow P A new epidemic phage type of *Staphylococcus aureus* 2 Characteristics of staphylococci lysed by phage 6557 Acta path microbiol scandinav 73 160-175 1968b

Bülow P A new epidemic phage type of *Staphylococcus aureus* 3 Occurrence and spread of Type 6557 with special reference to the consumption of some antibiotics Acta path microbiol scandinav 75 543-580 1968c

Cohen L S Fekety F R & Cluff L E Studies of the epidemiology of staphylococcal infection 4 The changing ecology of hospital staphylococci New Engl J Med 266 367 372 1962

Cohen L S Fekety F R & Cluff L E Studies of the epidemiology of staphylococcal infection 5 Infections in the surgical patient Ann Surg 159 321-334 1964

Comtois R D Untypable staphylococci lysis by experimental phages and other characteristics Canad J publ Hlth 56 199 204 1965

Comtois R D & Bynoe E T Report on a particular phage type of staphylococcus isolated in some hospitals in Canada Canad J publ Hlth 54 357-363 1963

Dolman C E Staphylococcus antitoxic serum in the treatment of acute staphylococcal infections and toxaemias III When demonstrable staphylococcaemia is present Canad Med Ass J 31 130-135 1934

Duran Reynals F Tissue permeability and the spreading factors in infection Bact Rev 6 197 252 1942

Faber V Bülow P & Rosendal K To be published

Faber V Jessen O Rosendal K & Eriksen K R Staphylococcal bacteraemia clinical and bacteriological observations in 201 cases Brit med J II 1832-1836 1960

Farrer S & McLeod C Staphylococcal infections in a general hospital Amer J Hyg 7 38 58 1960

Fekety F R & Bennet I L jr Epidemiological virulence of staphylococci Yale J Biol Med 31 [illegible] 1959

Fineg [illegible] & [illegible] D W Enterocolitis due to phage type 54 staphylococci resistant to kanamycin neomycin paromomycin and chloramphenicol New Engl J Med 263 1110 1116 1960

Finland M & Jones W F Staphylococcal infections currently encountered in a large municipal hospital some problems in evaluating antimicrobial therapy in such infections Ann N Y Acad Sci 65 191 20[illegible] 1956

Gillespie E H An outbreak of staphylococcal food poisoning Mth Bull Minist Hlth Lab Serv 6 8 10 1947

Harrell W E & Brown A E The treatment of septicaemia J Amer med Ass 116 [illegible] 183 1941

Hassall J E & Rountree P M Staphylococcal septicaemia Lancet 1 213 217 1959

Hay D R Staphylococcal septicaemia and pyaemia Quart J Med (N S) 29 313 331 1960

Hummel R P Hill E O & Altemeier W A Staphylococcal enterocolitis [illegible] in bacteriophage typing patterns J surg Res 3 289 292 19[illegible]

Jensen K Kombinationsbehandling med antibakterielle kemoterapeutika [illegible] kokinfektioner Ugeskr Læg 1[illegible] 1019 1030 19[illegible]

Jessen O Rosendal K Faber V Hove K & Eriksen

Staphylococcus aureus possibly related to pathogenicity 3 Bacteriological investigations of *Staphylococcus aureus* strains from 462 cases of bacteraemia Acta path microbiol scandinav 58 85–98 1963

Jessen O & Bulow P To be published

Jessen O Faber V Rosendal K Bulow P & Eriksen K R Changing staphylococci 2 A ten year study of bacteremia cases (To be published)

Keefer C Blake F G Marshall E K jr Lockwood J S & Wood W B jr Penicillin in treatment of infections report of 500 cases Statement by Committee on chemotherapeutic and other agents J Amer med Ass 122 1217–1224 1943

Knight V & Collins H S Current views on the problems of drug resistant staphylococci and staphylococcal infections Bull N Y Acad Med 31 549–568 1955

Kuipers A & Bennett N McK Staphylococcal septicaemia Med J Aust 1 217–222 1964

Lavine D Hurst V Grossman M & Lee M Staphylococcus of a newly recognized bacteriophage type Report of a hospital outbreak J Amer med Ass 193 935 938 1965

Lowenstein P S Staphylococcus septicaemia Amer J med Sci 181 196–203 1931

Mand H T H Staphylococcus septicaemia Arch Int Med 63 1068–1083 1939

Martin T D *Staphylococcus aureus* type 83A as a cause of hospital cross infection J Hyg (Lond) 61 499–505 1963

MacNeal W J & Frisbee F C One hundred patients with staphylococcus septicaemia receiving bacteriophage service Amer J med Sci 191 179–195 1936

Minchew B & Cluff L Studies of the epidemiology of staphylococcal infections 1 Infection in hospitalized patients J chron Dis 13 354–373 1961

Mitchell A A B New epidemic strain of *Staphylococcus aureus* Emergence and spread in a hospital Lancet 1 859–862 1964

Neuhof H Aufses A H & Hirschfeld S Pyogenic sepsis survey of 150 cases Surg Gynec & Obstet 58 886–896 1934

Parker M T Tomlinson A J H & Williams R E O Impetigo contagiosa The association of certain types of *Staphylococcus aureus* and *Streptococcus pyogenes* with superficial skin infection J Hyg (Lond) 53 458–473 1955

Powell D E B Staphylococcal septicaemia in a general hospital Brit med J 2 336–339 1961

Rosendal K Correlation of virulence of staphylococci from patients with bacteraemia with various laboratory indices Recent Progr Microbiol VIII Internat Congr Microbiol Montreal 1962 p 570–574

Rosendal K & Bülow P *Staphylococcus aureus* strains isolated in Danish hospital 2 From January 1st 1961 to December 31st 1965 Acta path microbiol scandinav 71 422 438 1967

Rosendal K & Jessen O Epidemic spread of *Staphylococcus aureus* phage type 83A Acta path microbiol scandinav 68 571–576 1964

Rosendal K Stenderup A Helms P & Eriksen K R *Staphylococcus aureus* strains isolated in Danish hospitals from April 1 to December 31st 1960 Acta path microbiol scandinav 58 72 84 1963

Rosenow F D & Brown A E Septicaemia a review of cases 1934 1936 inclusive Proc Mayo Clin 13 89–93 1938

Rountree P M & Beard M A The spread of neomycin resistant staphylococci in a hospital Med J Aust 1 498–502 1965

Scarpellino O Staphylococcus septicaemia South med J 32 173 1939

Schirger A Martin W J & Nichol D R Micrococcal bacteraemia without endocarditis clinical data and therapeutic considerations in 109 cases Ann intern Med 47 39–48 1957

Schmith H Eriksen K R & Rosendal K Impetigo Ugeskr Læg 119 1383 1392 1957

Scott W J M The principle of t[illegible]tment of septicaemia J Amer med Ass 105 1246 1249 1935

Shooter R A Smith M A G[illegible] J D Bro[illegible] M I A Williams R E O & Jevons M P Sprea[illegible] of [illegible] in surgical ward Brit med J 1 607–613 1958

Sboni K Behandling of gen[illegible] de taf[illegible] infektioner med dimethoksifenylpenicillin Ugeskr L[illegible] 1[illegible] 1136–1138 1962

Skinner D & Keefer C S S[illegible] of bact[illegible]ed by *Staphylococcus aureus* Arch Intern Med 6[illegible] 8[illegible]5 1941

Spink W W The clinical problem of antimicrobial resistant staphylococci Ann N Y Acad Sci 65 175–190 1956

Spink W W & Hall W H Penicillin therapy at the University hospitals 1942–1944 Ann intern Med 22 510–525 1945

Spittlehouse K F Phage types of *Staphylococcus pyogenes* isolated from impetigo and sycosis barbae Lancet 2 378 1955

Tisdale W A Fenster L F & Klatskin C Acute staphylococcal enterocolitis complicating oral neomycin therapy in cirrhosis New Engl J Med 263 1014–1016 1960

T e Ying T & Schu Chen H Staphylococci septicaemia an analysis of 116 cases Chin med J 75 217–232 1957

Waisbren B A & Abboud F Bacteraemia due to coagulase positive *Staphylococcus aureus* Ann intern Med 53 643–667 1960

Wallmark C & Finland M Phage types and antibiotic susceptibility of pathogenic staphylococci J Amer med Ass 1 896–897 1961

Williams R F O Investigations of staphylococcal infections acquired in Great Britain s hospitals Publ Hlth Rep (Wash) 73 961–970 1959

Williams R F O Ecological evidence for the existence of virulent strains of staphylococci Recent Progr Microbiol VIII Internat Congr Microbi l Montreal 1962 p 551 560

Wilson R & Hamburger M Fifteen years experience with staphylococcus septicemia in a large city hospital Ann J Med 22 437 457 1957

Acta path. microbiol. scandinav. 74: 573–590, 1968

The National Bacteriological Laboratory, Stockholm, Sweden

INVESTIGATIONS ON THE ENZYMES AND TOXINS OF STAPHYLOCOCCI

Study of Phosphatase Using p-Nitrophenyl Phosphate as Substrate

By

M. O. TIRUNARAYANAN

Received 14 v 68

The occurrence of the enzyme phosphatase in staphylococci was demonstrated in 1932 by *Gordon & Cooper* (10). Since that time staphylococcal phosphatase was studied for various reasons: from purely biochemical interest (4, 21, 27), to possible application of the phosphatase test to differentiate micro-organisms (5), to demonstrate the pathogenicity of staphylococci (1, 2, 8, 13, 19, 22, 24, 28, 31) as well as to study its relationship to phage type or penicillin susceptibility (6, 9, 18, 23). The relative ease with which the presence of phosphatase could be demonstrated by the agar plate method of *Bray & King* (5) made it possible for several laboratories to adopt this technique and use it as a good substitute for the coagulase test which was more cumbersome to perform. The investigations mentioned above were not extended further to evaluate the antigenic properties or its immunogenic role in staphylococcal infections. Coagulase and phosphatase, at one time thought to represent two functions of the same protein (14), were found to be separable entities (26).

For a number of reasons it seems possible that the different workers who studied the phosphatase reaction in fact investigated different enzymes. Apart from the use of different phosphate esters as substrates, the pH selected as optimal for the reaction have varied considerably from values of pH 10 down to pH 5.6. Further, the production of at least two phosphatases was borne out by the investigation of *Boivin & Mesrobeanu* (4), *Ohsaka, Mukai & Laskowski* (20), *Shah & Bloom* (26) and *Tirunarayanan & Lundbeck* (29). These results indicated that a proper characterization of the phosphatases produced by staphylococci was needed before any attempt to evaluate the significance of phosphatase activity or to correlate it with other properties of the bacteria could be made. An investigation was therefore undertaken to study the properties of the enzymes produced by 400 clinical strains using p-nitrophenyl phosphate as the substrate. The results are reported in the present communication.

MATERIAL AND METHODS

Strains and culture supernatants The strains and the culture supernatants were the same as those described earlier (16) The bacteria were stored in the lyophilized state and taken up on nutrient agar plates when needed They were then subcultured into Difco brain heart infusion broth to study phosphatase production

Assay of phosphatase For the study of phosphatase activity 0 002 M p nitrophenyl phosphoric acid di sodium salt (Hopkin and Williams Essex England) was used Incubation was carried out for 60 minutes at 37 C after the addition of the enzyme The liberated p nitrophenol was measured at pH 11 at 400 mμ using a Bausch & Lomb Spectronic-20 instrument using a control without enzyme as the blank When buffers of acid pH values were used the amount of 0 01 N NaOH was not sufficient to shift the pH value to 11 00 0 1 N NaOH was therefore used under such circumstances Enzyme reactions were also studied after incubation for different periods of time The rate was linear up to three hours incubation when the absorbance values did not exceed 1 0

For the routine assay of phosphatase activity incubation was done at two pH values pH 5 95 and pH 6 90 0 2 M sodium acetate acetic acid buffer of pH 5 95 and 0 2 M Tris HCl buffer of pH 7 00 (at 25 C) were used The supernatants were adjusted to the pH of the reaction mixture before being added The reaction mixture for these determinations consisted of

0 1 ml of 0 1 M $MgCl_2$ in distilled water
2 2 ml of buffer of desired pH
0 6 ml of 0 01 M substrate in distilled water
0 1 ml supernatant as enzyme source or purified enzyme

After incubation 3 ml of 0 1 N or 0 01 N NaOH was added before measuring the absorbance of the liberated p nitrophenol One micromole per ml of p nitrophenol gave an absorbance of 12 at pH 11 00 From the absorbance values the amount of p nitrophenol formed by one ml of enzyme preparation was [illegible]

In experiments where different pH values were required [illegible] tubes in pH activity relationship 0 9 per cent NaCl in distilled water [illegible] for the buffer First the components of the reaction mixture were [illegible] 4 C then mixed to give about 120 ml and the solution was rapidly adjusted [illegible] different pH values 3 ml aliquots of the reaction mixtures of different pH values [illegible] pipetted out into tubes and then placed in a water bath for incubation [illegible] pH [illegible] checked again after the incubation period before adding sodium hydroxide [illegible] The pH values were found to be the same before and after the incubation [illegible] However slight alterations were noted at the extreme pH values [illegible] more than 0 1 pH unit The use of a Radiometer pH titrator [illegible] Metrohm Dosimat of Metrohm which delivered either 0 1 N NaOH or 0 1 N HCl [illegible] to attain the set pH values made rapid adjustments of pH possible The total quantity of titrant needed did not exceed 2 per cent of the volume [illegible] mixture Similar pH adjustments were made while the effect [illegible] EDTA and metal ions such as beryllium was studied Although buffers [illegible] used these substances shifted the pH value to a great extent Experiments [illegible] at different ionic strengths using NaCl showed no striking difference in the phosphatase activity between concentrations of 0 0[illegible] M and 0 30 M of NaCl At [illegible] 10 per cent inhibition was obtained at 0 50 M and 50 per cent at 1 00 M NaCl [illegible] high salt concentrations were never reached during the pH adjustments

EXPERIMENTAL

Relationship of pH to Phosphatase Activity

Phosphatase activity at various pH values was studied in order to find whether differences between different strains existed and to study whether more than one enzyme was produced Supernatants of forty clinical strains were studied The activity curves for some of the strains shown in Fig 1 were found to differ from strain to strain Two

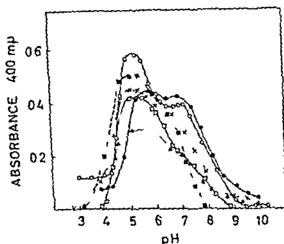


Fig 1

pH activity relationship for phosphatase produced by different strains of *Staphylococcus aureus*

strain no 17 □——□ strain no 7 ■- -■ strain no 116 ○——○
strain no 20 × - × strain no 77 ●- —● strain no 197 ▲ ▲

regions of peak activity could be demonstrated for some of the strains one having a maximum at pH values between 5 00 and 5 50 and another between pH 6 70 and 7 10 Different strains showed different proportions of the two activities The existence of the two pH optima was also described earlier (29) In the experiments described by *Barnes & Morris* (3) the pH activity curve showed also a notch around pH 7 10

Effect of Enzyme Inhibitors and Metal Ions on Phosphatase Activity

Because the pH activity curves seemed to indicate the presence of more than one enzyme in some of the preparations experiments were made to find whether one of the activities could be selectively inhibited This would provide additional support for the existence of more than one enzyme The enzyme preparations used for these studies were first purified by chromatography on Sephadex gel G 100 and Sepharose gel 4B as described elsewhere (29 30) Phosphatase activity was located in the void volumes on both the gels Thus the enzyme preparation was free of substances having molecular weights lower than 100 000 and 500 000 respectively The preparation from the Sepharose 4B column was also free of any esterase or lipase activities However variable quantities of magnesium apparently bound to the enzyme protein occurred in the different preparations as judged by the activity without added magnesium Attempts to remove such bound magnesium either by dialysis electrophoresis or ion exchange chromatographs resulted in considerable losses of activity Precipitation at pH 3 9 did not release the bound magnesium Thus the preparations from the

TABLE 1

Effect of some Enzyme Inhibitors on the Phosphatase Activity at pH 5.25 and at pH 6.90
(Values Expressed in Per Cent Residual Activity)

Inhibitor	pH 5.25					pH 6.90				
	10^{-1} M	10^{-2} M	10^{-3} M	10^{-4} M	10^{-5} M	10^{-1} M	10^{-2} M	10^{-3} M	10^{-4} M	10^{-5} M
NaF	43	91	90	96	105	3	21	49	103	106
EDTA	0	27	48	107	113	0	2	2	18	40
1 10 phenanthroline		39	54	74	85		165	107	82	71
KCN	0	41	100	100	109	0	38	101	105	104
Pyrophosphate		21	31	103	118	–	0	39	79	89
HOH		111	114	105	105		112	108	106	106
D tartaric acid		115	118	118	120	–	96	103	103	104
Lactic acid		71	89	100	100		97	100	107	101
L cysteine		11	46	77	81		4	100	104	105
Iodoacetamide		57	100	100	100		–	96	100	101

columns were used without adding any magnesium and without any further treatment Enzyme preparations from the following strains were used

strain no	phage pattern (RTD)
17	42D
7	75 77
18	81 47 83A 42D
14	NT (1000 RTD)
28	NT (1000 RTD)
44	6 47 53

No qualitative differences in the reactivity of the enzyme preparations from either gel from the same strain or other strains to the inhibitor compounds or metal ions could be established although slight quantitative differences occurred probably due to the different amounts of bound magnesium occurring in the enzyme

The effect of a number of established enzyme inhibitors on the phosphatase activity of the preparation from strain no 7 at the two pH values was studied and is shown in Table 1 The inhibitor compounds could be classified into many groups NaF EDTA and Na pyrophosphate inhibited the activity at pH 6 90 more than at pH 5 25 EDTA was the most potent 10^{-4} M causing 82 per cent inhibition at pH 6 90 while the activity at pH 5 25 was unaffected In fact a slight activation was noted but this was not reproducible in different preparations of the same strain Ethanol tartaric acid and lactic acid (hydroxyl compounds) did not affect the activity to any great extent L-Cysteine and iodoacetamide seemed to inhibit the activity at both pH values similarly at concentrations of 10 M or above Cyanide was similar to cysteine and iodoacetamide On the other hand 1 10 phenanthroline hydrochloride inhibited the activity at pH 5 25 and activated that at pH 6 90

The effect of some metal ions on the phosphatase activity at pH 5 25 and at pH 6 90 was studied As shown in Table 2 strontium and barium (as chlorides) diminished the activity of the enzyme at both pH values Manganese (chloride) inhibited the activity more at pH 6 90 than at pH 5 25 Zinc and nickel (sulphates) showed an activation at pH 5 25 and inhibition at pH 6 90 Copper (sulphate) was the most active and it also showed a stimulation of activity at pH 6 90 Beryllium (sulphate) gave inhibition of activity at pH 5 25 more than at pH 6 90

These results on the one hand with EDTA and phenanthroline on the other with beryllium copper and other metal ions showed that the activities at pH 5 25 and pH 6 90 could be differentiated Further studies were therefore made with these substances using concentrations more close to each other than the ten fold differences indicated in Tables 1 and 2

Studies with EDTA and metal ions The effect of different concentrations of EDTA on the activity at pH 5 25 and pH 6 90 was studied

TABLE 2

Effect of Metal Ions on the Activity of Phosphatase at pH 5.25 and 6.90
(Values Expressed in Per Cent Residual Activity)

Metal Ion	pH 5.25					pH 6.90				
	10^{-1} M	10^{-2} M	10^{-3} M	10^{-4} M	10^{-5} M	10^{-1} M	10^{-2} M	10^{-3} M	10^{-4} M	10^{-5} M
Sr	40	58	73	74	85	10	56	89	97	97
Ba	50	61	63	65	78	14	83	92	98	99
Zn		150	153	155	145	–	33	66	94	111
Mn		105	83	95	100	–	41	39	71	70
Ni		–	177	168	157		–	88	97	98
Cu		250	280	380	315		220	265	127	103
Be		0	14	58	77	–	41	79	73	90

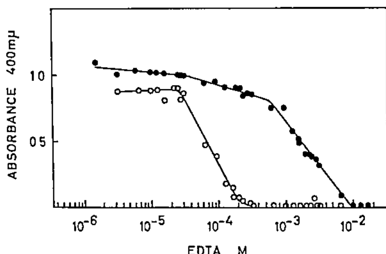


Fig. 2

Effect of EDTA on the phosphatase activity of strain no. 44 at pH 5.25 (●——●) and at pH 6.90 (○——○)

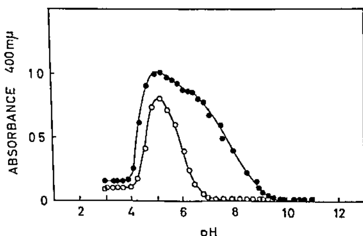


Fig. 3

pH activity curve for phosphatase from strain no. 44 in the presence of 3×10^{-4} M EDTA (○——○) and without EDTA (●——●)

and is shown in Fig. 2. Activity at pH 6.90 was completely inhibited at EDTA concentration of 2.8×10^{-4} M. Slight inhibition in this range was also noted at pH 5.25 but the major part of the activity was inhibited above 8×10^{-4} M of EDTA. These results showed that part of the enzyme activity was selectively inhibited by EDTA of lower concentrations. The pH activity curves of an enzyme preparation with and without the addition of 3×10^{-4} M EDTA were therefore compared (Fig. 3). The activity at pH 6.90 was completely inhibited and

TABLE 3

Effect of Metal Ions on EDTA Inhibition of Staphylococcal Phosphatase
(Value Expressed in Per Cent Residual Activity)

Metal ion	pH 5.95						pH 6.90					
	10^{-1} M	10^{-2} M	10^{-3} M	10^{-4} M	10^{-5} M	0†	10^{-1} M	10^{-2} M	10^{-3} M	10^{-4} M	10^{-5} M	0†
Sr	7.8	4.9	4.9	3.9	4.9	9.8	8.7	0	0	0	0	0
Ba	6.7	8.3	7.5	10.0	10.0	8.3	9.0	0	0	0	0	0
Ca	27.8	50.9	3.7	2.6	1.9	1.9	12.5	0	0	0	0	0
Mg^{++}	62.0	118.0	90.7	73.2	8.5	2.8	110.0	50	6.7	0	—	0
Mn	99.5	7.0	6.3	8.5	4.9	7.0	96.7	0.8	0.8	0.8	0	0
Zn	3.3	10.8	8.3	10.0	10.8	8.3	2.4	0	0	0	0	0
Cu	11.3	8.5	5.0	6.3	5.6	11.3	2.7	1.8	0	0	0	0.9
Ni		7.3	4.3	3.9	2.7	4.6		11.8	3.7	2.8	2.8	1.9
Be	–§	4.0	3.6	3.1	3.1	3.6	–§	–§	2.7	1.8	0	1.8

Values uncertain due to colour of metal ions § precipitation
† EDTA concentration at pH 5.95 was 1×10^{-2} M and at 6.90 was 1×10^{-3} M No magnesium was added.

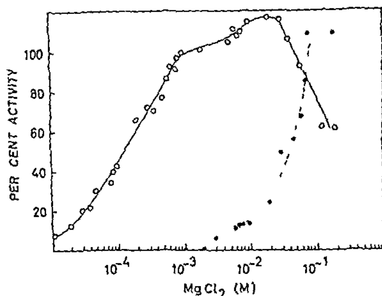


Fig 4

Reversal of EDTA inhibition of phosphatase by magnesium
pH 5 25 ○——○ pH 6 90 ●- - -●

the enzyme curve with EDTA showed thus the existence of only one component with a peak activity at pH 5 25

It seemed possible that different metal ions or different quantities of the same metal ion were needed for maximal activity at the two pH values Phosphatase preparations were therefore incubated first with 1 × 10 M and the effect of the addition of different metal ions in varying amounts on the activity was studied Results of studies done with the enzyme prepared from strain no 44 are outlined in Table 3 The activity was measured at pH 5 25 Similar studies done at pH 6 90 contained 1 × 10⁻³ M EDTA The results showed that Sr^{++} Ba^{++} Zn^{++} Mn Ni Cu^{++} and Be ions were practically inactive Mg alone activated to yield 100 per cent or more of the original activity 10⁻³ M Mg counteracted completely the EDTA inhibition at pH 5 25 whereas 10⁻¹ M was needed to show similar activation at pH 6 90 The activity with Ca^{++} was considerably lower than that of Mg

In order to find the optimum amount of magnesium needed to give maximal activity using the two different EDTA concentrations at the two pH values the concentration of magnesium was increased step wise Activity at pH 5 25 increased as the magnesium content was increased from 1 × 10⁻⁵ to 1 × 10⁻³ M reacing about 100 per cent of the original activity of the enzyme (Fig 4) Further increase in magnesium concentration increased the activity giving 120 per cent activity at 1 × 10⁻² M Still higher concentrations of magnesium showed an inhibition of the reaction At pH 6 90 the quantity of magnesium

1×10^{-3} M beryllium shifted the pH optimum to 6.20 but the total activity was not affected. Further shifts in pH optima up to 6.90 with 2, 3 and 4×10^{-3} M was noticed and the activity also diminished. These results indicated that beryllium affected the enzyme activity at pH 5.25 considerably more than that at pH 6.90. Experiments on the effect of different amounts of magnesium and calcium ions on beryllium inhibition of the phosphatase showed that magnesium counteracted the inhibition whereas calcium was without effect (Table 4). With magnesium, however, complete reversal of the inhibition at pH 5.25 was not obtained.

TABLE 4

Effect of Calcium and Magnesium on the Inhibition of Staphylococcal Phosphatase by $BeSO_4$ (Values in Per Cent Residual Activity)

		pH 5.25	pH 6.90
Control without $BeSO_4$		100	100
$BeSO_4$		18.8	0
$BeSO_4$ + CaCl	1×10^{-5} M	12.3	0
	1×10^{-4} M	12.6	0
	1×10^{-3} M	12.3	0
	1×10^{-2} M	11.5	0
	1×10^{-1} M	13.1	0
$BeSO_4$ + MgCl	1×10^{-5} M	38.8	1.6
	1×10^{-4} M	27.5	53.6
	1×10^{-3} M	28.6	69.4
	1×10^{-2} M	52.2	72.6
	1×10^{-1} M	80.8	114.5

Concentration of $BeSO_4$ 0.0016 M at pH 5.25 and 0.015 M at pH 6.90

Effect of phenanthroline and copper ions on phosphatase. It was observed that phenanthroline depressed the activity at pH 5.25 while it stimulated that at pH 6.90 (Table 1). Experiments with various amounts of phenanthroline showed that the activation at pH 6.90 was maximal at concentrations between 1 and 3×10^{-4} M. The pH activity curves with and without phenanthroline are shown in Fig. 8. It was found that the addition of 2×10^{-4} M phenanthroline increased the activity of the enzyme almost twofold at pH 6.90. The inhibition at pH 5.25 noted in Table 1 was only apparent due to the shift in the pH optimum.

Phenanthroline is a chelating agent which forms ready complexes with copper and iron. It seemed possible that either of these or other metal ions was inhibitory to the activity at pH 6.90 which was removed by phenanthroline. On the other hand the addition of copper sulphate to the enzyme preparation gave a similar increase in activity but with the pH optimum around 5.25 (Fig. 8). 1×10^{-4} M copper gave the maximal activity. Higher amounts showed an inhibition. Thus the activity at pH 5.25 was stimulated by copper and inhibited at pH 6.90.

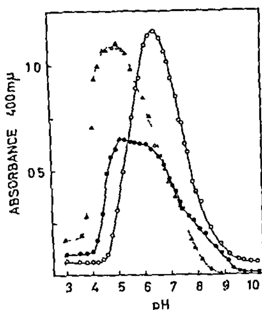


Fig 8

Effect of 1 10 phenanthroline and $CuSO_4$ on the phosphatase of strain no 28
Control ●——● 2 × 10⁻² M phenanthroline ○——○ 1 × 10⁻⁴ M $CuSO_4$ △ - - △

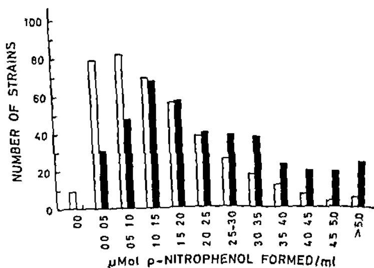


Fig 9

The production of phosphatase by 400 clinical strains
■ activity at pH 5 25 □ activity at pH 8 90

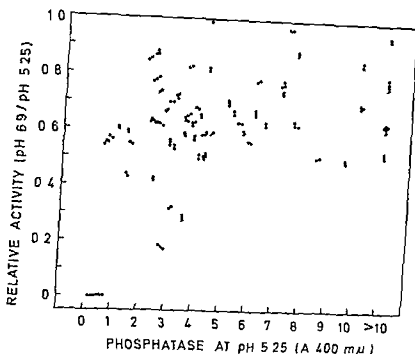


Fig. 10
Correlation between the phosphatase activity of clinical strains at pH 5.25 and pH 6.90

Production of Phosphatase by Clinical Strains

Supernatants of 400 clinical strains were analysed for the content of phosphatase measuring the activity at pH 5.25 and pH 6.90. The object of these studies was to select strains which produced large amounts of the enzymes which might be used in subsequent vaccine production. In addition it was also of interest to find if strains occurred which produced predominantly one kind of the enzyme.

The activity of phosphatase (μMol p-nitrophenol formed per ml supernatant per hour) was plotted against the number of strains giving the reaction (Fig. 9). When the activity was measured at pH 6.90 it was observed that ten strains did not give any reaction. 298 strains (75 per cent) gave low activity (0.0 to 2.0) while 92 strains (23 per cent) showed high amounts of phosphatase. Only five strains produced as high amounts of the enzyme as >5 μMol. Tests made at pH 5.25 showed that all 400 strains were phosphatase producers. 240 strains (60 per cent) gave low activity (0.0 to 2.0) while the rest produced higher amounts of the enzyme. 21 strains produced more than 5 μMol p-nitrophenol. The number of strains giving activity at pH 6.90 were consistently higher than those giving activity at pH 5.25 up to p-nitrophenol values of 1.5 μMol. On the other hand the relation was the opposite with activity above 1.5 μMol.

Phosphatase activity was determined concurrently at both pH values using the same supernatants. It was of interest to find the relative proportion of the activity at pH 6.90 to that at pH 5.25 at various levels of activity. Any correlation observed would point to the identity of the two activities provided only one enzyme was involved. Such an attempt to correlate the activities is represented in Fig. 10. It was observed that the enzyme activity at pH 6.90 varied considerably and was not correlated to the activity at pH 5.25. These results point to the possibility that the two activities might represent two different enzymes particularly as all the strains were grown in the same medium and contained the same amounts of the different metal ions.

DISCUSSION

The results on the phosphatase activity of the culture supernatants of staphylococci indicated that p-nitrophenyl phosphate was hydrolysed optimally at two different pH values, pH 5.25 and pH 6.90. No other pH optimum was demonstrable among the strains investigated in the present study. This was surprising considering the reports in the literature where several pH optima were noted as indicated below.

pH optimum	substrate used	investigators
10.00	acid insoluble-P	*Brown & Mesrobeanu* (4)
9.50	fructose-1,6-diP	*Strasters & Winkler* (27)
9.10	p-nitrophenyl-P	*Shah & Blobel* (26)
9.00	p-nitrophenyl-P	*Ohsaka et al.* (20)
8.00 – 8.70	β-glycero-P	*Cardon & Cooper* (10)
7.40	phenolphthalein-P	*Bray & King* (5)
7.20	p-nitrophenyl-P	*Inniss & San Clement* (14)
5.60 – 7.00	α-glycero-P	*Fay? & Vittu* (21)
6.00	phenolphthalein-P	*Cann & Hawn* (6)
6.00	acid soluble-P	*Brown & Mesrobeanu* (4)
5.60	p-nitrophenyl-P	*B[illegible] & M[illegible]* (3)
5.50	p-nitrophenyl-P	*Ohsaka et al.* [illegible]

The use of different substrates may account for the variation of the pH optima. With p-nitrophenyl phosphate as the substrate, the pH optima were described to be 5.50, 5.60 (3, 20), 7.20 (14) and 9.00, 9.10 (20, 26). The first two values agreed closely with the investigations reported in this study, whereas no activity was found around pH 9.00. A careful study of the results obtained by *Ohsaka et al.* (20) showed that the activity of alkaline phosphatase was very low giving 8 × 10 units at pH 9.00, whereas the corresponding activity at pH 5.60 gave 2.5 × 10 units. Thus the content of alkaline phosphatase was about a hundredth of the acid phosphatase. This made it difficult to demonstrate with certainty the alkaline phosphatase activity in the supernatants. Further, *Shah & Blobel* (26) showed that alkaline phosphatase production is inhibited when the bacteria are grown in the presence of inorganic phosphate as done in the present study.

The two pH optima the different proportions of the two activities produced by clinical strains as well as their different reactivity to metal ions and metal chelating agents point to the possibility that two phosphatases may be involved Certain observations made in the present study are of interest from this point of view Thus heavy metal ions like copper stimulated the activity at pH 5 25 Phenanthroline showed the opposite effect as it stimulated phosphatase at pH 6 90 The activation of the phosphatase at pH 5 25 by copper did not occur when magnesium was removed by EDTA Apparently a copper magnesium complex was needed for the hydrolysis of this substrate Whether this was bound to the substrate or the enzyme protein cannot be stated with certainty On the other hand the quantity of magnesium needed for the activity at pH 5 25 was considerably lower than that required for activity at pH 6 90 Thus larger amounts of EDTA and lower amounts of beryllium were needed to inhibit the activity at pH 5 25

Whereas the above results point to the possibility of two phosphatases being involved the occurrence of a single enzyme showing different pH optima depending upon metal ion activator or inhibitor cannot be overruled For example arginase was found to exhibit a pH optimum around 10 with Mn^{++} and 7 with Co^{++} and Ni (11) Thus on the basis of these results the existence of two phosphatases in the culture supernatants of staphylococci cannot be stated with certainty

SUMMARY

An analysis of the phosphatase activity in the culture supernatants of 400 clinical strains was made Two pH optima (pH 5 25 and pH 6 90) were noted for the hydrolysis of p nitrophenyl phosphate which was used as the substrate The two activities occurred in the different supernatants in varying amounts indicating no correlation between each other Differences were also found in the response to metal ions and metal chelating agents Copper activated the reaction at pH 5 25 and phenanthroline counteracted this effect EDTA inhibited the enzyme activity at pH 6 90 more than at pH 5 25 whereas beryllium showed the opposite relation Although these results point in favour of the existence of two phosphatases in the supernatants of staphylococci the existence of a single enzyme with different reactivity to metal ions and showing different pH optimum depending on such ions seemed possible

REFERENCES

1 *Barber M Brocksbank B W L & Kuper S W A* Staphylococcal phosphatase glucuronidase and sulphatase *J Path Bact* 63 57 64 1951
2 *Barber M & Kuper S W A* Identification of *Staphylococcus pyogenes* by the phosphatase reaction *J Path Bact* 63 65–68 1951
3 *Barnes F H & Morris J F* A quantitative study of the phosphatase activity of *Micrococcus pyogenes* *J Bact* 73 100 104 1957

4 *Boivin A & Mesrobeanu I* Contribution à l'étude de la composition chimique des bactéries Le substances phosphorees au cours de l autolyse bactérienne. Compt rend soc biol *112* 611-613 1933

5 *Bray J & King E J* The phosphatase reaction as an aid to the identification of microorganisms using phenolphthalein phosphate as substrate J Path Bact 55 315-320 1943

6 *Cannon F D & Hawn C V Z* Phosphatase activity of *Staphylococcus aureus* Correlation of enzyme production with resistance to penicillin and phage pattern J Bact. *86* 1052-1056 1963

7 *Capellato M* Attività fosfatasica negli schizomiceti Boll Soc ital Biol sper 22 89-91 1946

8 *Carrère L & Roux J* Activité phosphatasique des staphylocoques Ann Inst Pasteur *87* 349-353 19 4

9 *Cillissen G & Rula M* The correlation between phosphatase activity and resistance to penicillin in *Micrococcus pyogenes* Zbl Bakt I Orig *171* 281-285 1958

10 *Gordon J & Cooper K E* A study of the phosphorus distribution in bacterial cultures III Phosphatase activity in *B coli* and *Staphylococcus* Brit J Exp Path *13* 503 508 1932

11 *Greenberg D M* Arginase In The Enzymes ed Boyer P D Hardy H & Myrba k K Academic Press Inc *4* 257-267 1960

12 *Grier R S Hood M B & Hoagland M B* Observations on the effects of beryllium on alkaline phosphatase J Biol Chem. *180* 289-298 1949

13 *Gupta S P & Chakravarti R N* Observations on some biochemical reactions of staphylococci with special reference to coagulase and phosphatase production tests Indian J Med Res *42* 131-136 1954

14 *Inniss W B & San Clemente C I* Biochemical studies on staphylocoagulase and an allied phosphatase activity J Bact *83* 941-947 1962

15 *Klemperer F W Miller J M & Hill C J* The inhibition of alkaline phosphatase by beryllium J Biol Chem *180* 281-288 1949

16 *Lundbeck H & Tirunarayanan M O* Investigations on the enzymes and toxins of staphylococci Study of the egg yolk reaction using an agar plate assay method Acta path microbiol scandinav *68* 123-134 1966

17 *Malveaux F J & San Clemente C L* Free and bound acid phosphatase in *Staphylococcus aureus* Bact Proceedings 44 1966

18 *Markov K I Saev G K Klein S & Monov V* Phosphatase formation by penicillin resistant and penicillin sensitive staphylococci Naturwissenschaften 45 443-444 1958

19 *Mohan R C Pradhan S K & Manjrekar P S* Pathogenicity of phosphatase positive but coagulase negative staphylococci Indian J Med Sci *9* 482-484 1955

20 *Ohsaka A Mukai J I & Laskowski Sr M* The use of purified micrococcal nuclease in identifying the nucleot de terminus bearing a free 5 mono phosphate J Biol Chem 239 3498-3504 1964

21 *Paget M & Vittu C* Recherches sur l système phosphom no te a q chez *Escherichia coli Staphylococcus aur us* et S *albus* C R Acad S (Paris) 223 216-218 1946

22 *Pal S C & Ray B G* Phosphat se act vity of pathogen c staphyl cci Indian J Med Sci *16* 516-519 1962

23 *Pan Y L & Blumenthal H J* C rrelation between acid ph osph at c and c a gulase production or phage t p of *Staphylococ i au u* J Bact *8* 124-129 1961

24 *Rangam C M & Kalda e S M* Pho phatase activity of [illegible] an indication of their pathog nicity Indian J Med S [illegible] 1954

25 *San Clemente C L & Zolli J Z* Separation of phosphata r m taphyl coagulase preparations J Bact *86* 606-608 1963

26 *Shah D B & Blobel H* Repres ible alkaline phosphata e f *Staphyl coc us aureus* J Bact. *93* 780-781 1967

27 *Strast r K O & Winkler K C* Carbohydrate metabol sm f *Staphyl coccus au us* J Gen Microbiol *33* 213-229 1963

28 *Staheli H* Die Pho ph ataseproduktion der Staphylokokken un I hre Beziehung zur Pathogenität Schweiz Z allg Path Bakt 15 26- 32 1952

29 *Tirunarayanan M O & Lundbeck H* Investigations on the enzymes and toxins of staphylococci Relationship of pH to growth and production of enzymes Acta path microbiol scandinav 74 274 286 1968

30 *Tirunarayanan M O & Lundbeck H* Investigations on the enzymes and toxins of staphylococci Separation of lipase from phosphatase and spectrophotometric analysis of the egg yolk reaction Acta path microbiol scandinav 73 429–436 1968

31 *White M L & Pickett M J* A rapid phosphatase test for *Micrococcus pyogenes var aureus* for detection of potentially pathogenic strains Amer J Clin Path 23 1181 1183 1953

Acta path. microbiol. scandinav. [illegible] 602, 19[illegible]

From the Department of the Central Bacteriological Laboratory of Stockholm City,
P.O. Box 1[illegible], Stockholm 1, Sweden.

STUDIES OF ECHOVIRUS TYPE 6 ANTIGENS IN IMMUNODIFFUSION

I. Antigen Composition of Native and Heated Virus Preparations

By

MARIANNE FORSGREN

Received [illegible] 68

During the last ten years, several enterovirus antigen-antibody systems have been analyzed with Ouchterlony gel double diffusion technique. The advantage of this technique is the possibility of obtaining qualitative information on complex antigen-antibody systems. It permits identification and comparison of different antigen-antibody systems. *LeBouvier* (1957) first demonstrated specific precipitates in agar between preparations of poliovirus and hyperimmune sera. Later *Le Bouvier* (1957, 1959 a, b), *Grasset et al.* (1958), *Paccaud et al.* (1960 a, b), *Balayan* (1960, 1961), *Selzer* (1961, 1962) and *Ohlson* (1963 a, b = present author) studied in greater detail reactions between poliovirus preparations and human convalescent sera, postvaccination sera and hyperimmune sera. Two different antigens are encountered in native preparations: the complete nucleic acid containing N (or D) antigen and the nucleic acid deficient H (or C) antigen. The two antigens have been separated by density gradient centrifugation (*[illegible] et al.* 1957, *Schwerdt* 1957, *Rotman et al.* 1958) and also in immunoelectrophoresis (*Ohlson* 1963). During recent years the coxsackieviruses have been studied in gel double diffusion by *Schmidt et al.* (1962 a, b, 1963, 1965 a, b). Analogous to the poliosystems two antigens were found: a more type specific N (D) and a group reacting H (C). The antigens were separated by density gradient centrifugation.

In early immunodiffusion studies of echoviruses only one precipitate was found whether animal hyperimmune sera (*Ohlson* 1963, *Middleton et al.* 1964) or even human convalescent sera (*Balayan et al.* 1961, *Middleton et al.* 1964) were employed. Later studies proved the existence of two antigens also for echoviruses, however, when the antigen preparations were tested against homologous convalescent sera (*Forsgren* 1966, echovirus 6; *Conant et al.* 1966, echoviruses 1 and 8; *Co*

This work was aided by a grant from the [illegible] Foundation. The skilful technical assistance of Mr. [illegible], Mrs. [illegible], Mrs. Astri Andersson and Mr. [illegible] is gratefully acknowledged.

nani et al (1966) reported that one of the echovirus 4 precipitates was type specific whereas the other one seemed to be common also to polio virus 2 and coxsackievirus B 5

In the present paper a full report is given on the author s immuno diffusion studies of echovirus 6

MATERIALS AND METHODS

Virus strains Echovirus 6 prototype strain d Amori adapted to HeLa cells Polio virus 1 Brunhilde Poliovirus 2 wild strain isolated in Stockholm (No 5365) Polio virus 3 Saukett All strains were checked in neutralization against NIH reference hyperimmune sera and in CF with guinea pig hyperimmune sera (kindly supplied by dr A Macrae Colindale London) for absence of contamination with other viruses employed in this study

Cell strain HeLa cells Outgrowth medium early in this investigation Parker 199 (*Morgan et al* 1950) + 40 per cent human serum was used Later the serum con centration was lowered to 20 per cent with equal results The strain and the antigens were continuously checked for absence of bacterial mycotic and mycoplasma con tamination (mycoplasma medium Difco PPLO broth + 20 per cent native horse serum incubation time 14 days)

Virus cultivation Stationary Roux bottle cultures of HeLa cells were washed 3 times with phosphate buffered saline (PBS) 40 ml Parker medium 199 was added and the culture was infected at a high input multiplicity (about 10) After 3 hours stationary incubation at 35 C the medium was removed and replaced with 15 ml fresh Parker medium The bottles were incubated at 35 C on a rocking table (12 cycles/hour) to allow the small amount of medium to nourish the cells. The de generation was complete after 24–48 hours at which time the fluid was harvested after 3 cycles of freezing and thawing It contained 10^9–10^9 $TCID_{50}$/ml and 16–64 CF antigen units

A few virus batches for immunization purposes were similarly prepared in pri mary cultures of cynomolgous monkey kidney cells

Preparation of Antigen for Gel Diffusion Studies

Infectious tissue culture fluid from HeLa cultures was centrifuged at 3 00 rpm in an angle centrifuge for 45 min In a Spinco model L the supernatant was further clarified by centrifugation at 10 000 rpm (rotor 30) for 30 min (average 8 × 10^3 g) and then spun at 30 000 rpm for 150 180 min (average 78 × 10^3 g) The pellet was resuspended in PBS Coarse material was spun down at 10 000 rpm for 5–15 min in Spinco (rotor 40) The supernatant was used as precipitating antigen and stored at —30 C During the whole preparation procedure the materials were kept at 0 C. The concentration factor was 100 times by volume for echovirus 6 25–50 for polio viruses

Antigen Preparations Used for Immunization

The antigens consisted of infectious tissue culture fluid prepared as above only the freezing and thawing procedure being omitted HeLa cell antigens were subjected to one cycle of freon treatment (ana partes infectious tissue culture fluid and freon 113 kept at 0 C) in a Servall omnimixer for 4 5 min at about 14 000 rpm By this procedure the main part of the tissue antigens was removed the infectivity however being retained (10^8 $TCID_{50}$ ml and 8 16 CF antigen units) (*Hamparian et al* 1958) For comparison a few materials prepared in monkey kidney cells were used without freon treatment

Guinea Pig Hyperimmune Sera

After a comparison of various immunization schedules (see Table 1) the follow ing procedure was adopted guinea pigs were immunized with 2 ml adjuvant—virus antigen mixture (ana partes) i m and 1 ml virus antigen i p (without adjuvant)

he adjuvant used was Arlacel A + paraffin oil The i.p injection (without adjuvant) was repeated 3 times at weekly intervals A booster injection was given 3–4 weeks after the fourth injection The animals were bled one week after the last injection Sera were stored at —30 C.

Human Sera from Patients with Echovirus 6 Infections

Acute and convalescent sera from patients with the diagnosis of echovirus 6 infections (echovirus 6 isolated from stool specimen and/or from cerebro spinal fluid titre rise or high titres of neutralizing antibodies in acute–convalescent sera) were used Many of the sera were collected during an epidemic in Stockholm in 1954 (*von Zeipel et al* 1957) Sera were inactivated at 56 C 30 min and stored at —20 C

Immunodiffusion Tests

A micromodification on slides was used ? ml of 1 per cent Behringwerke Reinagar in PBS A solution was poured on microscopic slides (26 × 76 mm) Wells were punched with cutters made of cannulae of the desired diameter a plexiglass template being used to guide the cutters The most frequently used template gave wells of a diameter of 4 mm 3 mm apart Primary agar coating of the glass slides was performed but this was omitted during the last phase of the investigation and no leakage phenomena have been observed

The reagents were added by means of disposable capillary pipettes A 4 mm well contained 0 025 ml and the amount filled was quite reproducible To diminish the risk of handling highly infectious material the plates were marked and placed in large Petri dishes before being filled Incubation was performed in a moistened chamber at room temperature (average 20 –25 C) Some precipitates did not require more than one day of incubation after two days all systems were clearly visible The slides were incubated for 7 days before being discarded Photographs were taken in dark field illumination after 1 ? and sometimes also after 7 days

No preservative was added to the agar

Nucleic Acid Staining with Acridine Orange

Staining of the RNA with acridine orange was performed according to the technique earlier described (*Ohlson* 1963) The excess of the reagents was removed by washing the slides with PBS The slides were fixed in Carnoy s fixative chloroform ethanol glacial acetic acid and then stained at pH 4 0 with 0 01 per cent acridine orange (Merck) Precipitates with the heat labile antigen showed a brilliant red fluorescence The heat stable antigen did not stain with acridine orange and showed no fluorescence

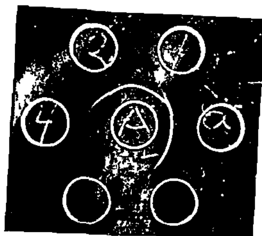
Complement Fixation Tests (CF)

The Fulton Dumbell plate technique as modified by *Sv dmyr et al* (1953) was used.

RESULTS

Precipitin Reaction with Human Sera

Native antigen preparations were tested against human acute and convalescent sera from patients with echovirus 6 infections With most acute sera a single line was obtained (see Fig 1) This line was not stained with the RNA staining acridine orange (AO) However with convalescent sera two separate lines were obtained (see Fig 2) One of the two precipitates showed red fluorescence after acridine orange staining while the other did not The line obtained with acute serum fused completely with the AO negative line obtained with convalescent serum (see Fig 4 s and s) When the same sera were tested against



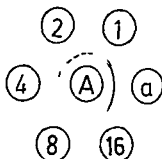
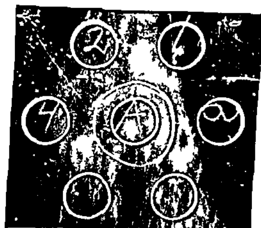


Fig 1 Acute phase serum



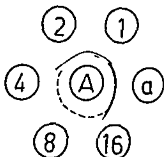


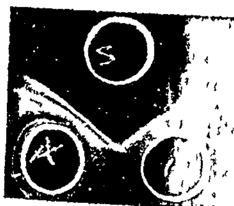
Fig 2 Convalescent serum from the same patient

Figs 1 and 2
Human serum precipitated with native echovirus 6 preparation
a guinea pig reference serum 1–16 dilutions of human serum
A native preparation containing N and H antigens
—— N antigen antibody precipitate showing red fluorescence with AO
- - - H antigen antibody precipitate showing no fluorescence with AO

heated (56 C 30 min) antigen material only one line was seen (se Fig 5 s_1 and s) This line was not stained with AO

A comparison of native and heated antigen preparations against human convalescent serum showed a reaction of identity between th two AO negative lines (see Fig 3) The AO positive line showed a re action of non identity with the AO negative line of the heated pre paration

Thus native preparations of echovirus 6 contain two different antigens one heat labile AO staining (N) and one heat stable AO negative antigen (H)



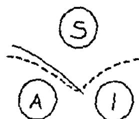


Fig
Compari on cf native and heated echovi ins against a human con valescent serum A native prepar tion c 3 H antigens I A heated at 56 C 30 min precipitating x H alescent serum containing antibodie against ens
—— N antigen antibody precipitat luorescence with AO
--- H antigen antibody precipitat luorescence with AO

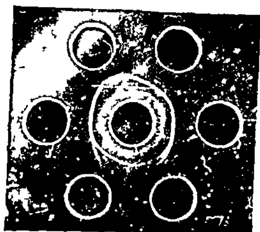
Precipitating antibodies against i ns appear in human sera during infection with echovirus 6 rises against one or both antigens are often obtained in conv t sera (compare Figs 1 and 2) A detailed paper on this subject published

Precipitin Reaction with Sera from *a Pigs Hyperimmuni ed with Native Echovirus 6 Preparation*

No precipitating antibodies were present in any normal guinea pig sera tested

As might be expected animals immunized with antigens in oil adjuvant showed the best antibody response (see Table 1) Accordingly sera from such animals were used in sub quent work Sera were inactivated at 56 C 30 min before use A comparison between native and heated sera did not show any difference in the results however

When tested against some native antigen batches sera from animals immunized with native echovirus 6 preparations gave rise to two separate lines one AO positive and one AO negative (see Figs 6-9) Most native antigen preparations however gave only one precipitate with the same sera (see Figs 1 2 and 4) This single precipitate was stained with acridine orange and fused completely with both the N and H precipitates obtained with human convalescent sera (see Fig serum a and Fig 4 sera s_1 and s) When compared to an H precipitat obtained with a human serum there was a fusion between the lin obtained with guinea pig serum and the human lin but a spur formation occurred (see Fig 1 serum a) The spur showed RNA reaction with AO A reasonable explanation would be that the



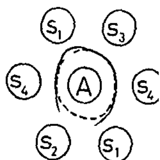
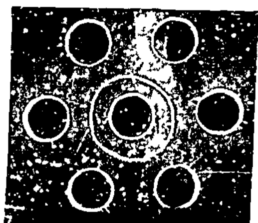


Fig 4 Native virus preparation



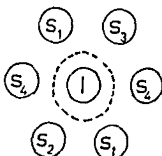


Fig 5 Heated virus preparation

Figs 4 and

Human sera and guinea pig anti echo 6 immune sera precipitated with echo virus 6 antigen preparations s_1 human convalescent serum containing antibodies active against N and H antigens s_2 human acute phase serum containing antibodies active against H antigen s_3 guinea pig hyperimmune serum against native echovirus 6 s_4 guinea pig hyperimmune serum against heated echovirus 6 A virus preparation containing N and H antigens I A heated at 56 C 30 min containing H antigen

—— N antigen antibody precipitate showing red fluorescence with AO

– – – H antigen antibody precipitate showing no fluorescence with AO

two antigen antibody precipitates (N and H) were superimposed on each other

When the same animal sera were tested against heated antigen (56 C 30 min) a single precipitate was always obtained This line was not stained with AO and fused completely with the H line obtained with human sera (see Fig 5 sera s_2 and s_1) A comparison between native and heated antigen preparations against hyperimmune serum was also done Again the AO negative lines fused completely (Fig 6)



Fig

Comparison of native and heat ... oviru ... 3 ... ur ... serum ... native virus prep ... c nt ... d H ar ... 56 C 30 min precipitating as ... sur ... immun ... echov r ... at

—— N antigen antib ... ipit ... s ... el ...
--- H antigen antib ... ipit ... sl ... in ... ne

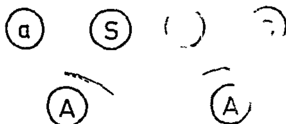


Fig 7 *Fig 8*

Figs 7 9

Absorption of guinea pig anti echo 6 immune serum with echo virus 6 preparation heated for 15 min (Fig 7) 30 min (Fig 8) and 60 min (Fig 9) at 56 C. s Unabsorbed guinea pig hyperimmune serum against native echo virus 6 containing antibodies against N and H antigens Serum s absorbed with virus preparation heated for a 15 min b 30 min c 60 min A Native virus preparation containing N and H antigens

—— N antigen antibody precipitate showing red fluorescence with AO
--- H antigen antibody precipitate showing no fluorescence with AO

Heating and Absorption Experiments

Echovirus 6 preparations were heated for 15 30 or 60 min at 56 C Precipitations with the different heated materials against one of the above mentioned hyperimmune sera were done The same heated preparations were also used in absorption experiments on the same serum For this procedure antigen and serum were mixed in equal proportions and filled into the cups without further incubation The following results were obtained heating of the virus preparation for 15 min resulted in loss of the AO positive component only in AO negative

TABLE 1

Guinea Pig Hyperimmune Sera against Native Echovirus 6

Animal No	Antigen prepared in	Log TCID$_{50}$/ml	No of injections	Adjuvant	Titre of precip antibodies	
					N	H
1 2	Monkey kidney cells	8 9–9 3	1 i m + i p —4 i p	—	8	8
3 4 5 6	Monkey kidney cells	8 9–9 3	1 i m + i p —4 i p	+	64–128	64–128
7 8 9 10	HeLa cells freon treated	7 7§	1 i m + i p —4 i p	+	128	128
11 12 13	HeLa cells freon treated	7 7§	1 i p	—	8	8

TABLE 2

Guinea Pig Hyperimmune Sera against Heated (30 56 C) Echovirus 6

Animal No	Antigen prepared in	Log TCID /ml	No of injections	Adjuvant	Titre of precip antibodies	
					N	H
14 15	Monkey kidney cells	0 05/ml	1 i m + i p —4 i p	—	4–8	8
16–17 18 19	Monkey kidney cells	0 05/ml	1 i m + i p —4 i p	+	32–64	64–128
20 21 22	HeLa cells freon treated	0 5/ml§	1 i m + i p —4 i p	+	32–64	64–128
23 24	HeLa cells freon treated	0 5/ml§	1 i p	—	8	8
25	HeLa cells freon treated	0 5/ml§	1 i p	—	2	2

Tested in monkey kidney cell cultures

§ Tested in HeLa cell cultures N titre lo s 1 s freon treatment (Table 1)



Fig 10
Guinea pig hyperimmune serum prepared with echovirus 6 heated at 56 C 30 min precipitated with echovirus 6 antigen preparation 16–64 serum dilutions A virus preparation containing N and H antigens
—— N antigen antibody precipitate showing red fluorescence with AO
--- H antigen antibody precipitate showing no flu rescence with AO

precipitate being observed However absorption of the serum with the same material resulted in absorption of antibodies against the N as well as the H antigen (see Fig 7) The preparation heated for 30 min also precipitated as H antigen only (see Figs 3 and 6) but absorbed anti bodies preferentially against the H antigen (see Fig 8) Heating for 60 min resulted in no or very weak precipitating H activity and this pre paration had no significant absorbing capacity (see Fig 9)

These absorption experiments demonstrate that also in guinea pig hyperimmune sera antibodies against the N and H antigens are present

Precipitin Reaction with Sera from Guinea Pigs Hyperimmunized with Heated Echovirus 6 Preparations

Guinea pigs were immunized with echovirus 6 preparations heated at 56 C 30 min The preparations were otherwise the same as those used above The remaining infectivity was low (at most 0 5 $TCID_{50}$/ml) Sera from these animals however showed antibodies not only against the H but also against the N antigen (see Table 2 and Fig 4 serum s_3 and Fig 5 s_4) Most of these animal sera when tested against native antigen preparations gave two precipitating systems not superimposed (Fig 10) These sera contained neutralizing antibodies to almost the same titres as the sera prepared against native echovirus 6

All hyperimmune sera were tested in immunodiffusion and comple ment fixation for antibodies against poliovirus antigens No antibodies against either the N or the H antigens of the three poliovirus types could be demonstrated

Reactions with tissue antigens did not interfere in the present ex